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"What you do makes a difference,

and you have to decide what kind of

difference you want to make."

~ Jane Goodall ~

Aos meus Pais

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

Manuscript A. Correia-Branco A, Azevedo CF, Araujo JR, Guimaraes JT, Faria A, Keating E and Martel F. **Xanthohumol impairs glucose uptake by a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) and impacts the process of placentation.** Molecular Human Reproduction, 2015, 21(10):803-815.

Molecular Human Reproduction (Mol Hum Reprod)
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Manuscript B. Correia-Branco A, Keating E and Martel F. **Involvement of mTOR, JNK and PI3K in the negative effect of ethanol and metformin on the human first-trimester extravillous trophoblast HTR-8/SVneo cell line**. European Journal of Pharmacology, 2018 (accepted).

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Manuscript C. Correia-Branco A, Keating E and Martel F. **Arachidonic Acid Reverses Xanthohumol-Induced Insufficiency in a Human First-Trimester Extravillous Trophoblast Cell Line (HTR-8/SVneo Cells).** Reproductive Sciences. 2017: 1933719117746762.

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Manuscript D. Correia-Branco A, Keating E and Martel F. **Placentation-related processes in a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) are affected by several xenobiotics.** Drug and Chemical Toxicology, 2018, 1-5.

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ABSTRACT

The placenta provides the interface between the fetal and maternal environments, exchanging gases, nutrients and waste products between the mother and the growing fetus, serving as an endocrine organ by producing several pregnancy-associated hormones and growth factors and protecting the fetus from maternal immune attack. Placentation – placenta formation and development - is a continuous and highly regulated process that initiates immediately upon fertilization and ends only after delivery. Extravillous trophoblasts (EVTs) are the key cells involved in the placentation process. They are fully specialized trophoblasts displaying an invasive and proliferative phenotype, which perform the anchorage of the chorionic villi into the uterine wall and actively regulate uterine spiral arteries remodeling, a process that ends up with the establishment of the utero-placental blood flow.

The major aim of this study was to investigate the impact of xenobiotics upon placental transport of important nutrients (glucose (GLU), the long-chain polyunsaturated fatty acids (LC-PUFAs) arachidonic (ARA) and docohexaenoic (DHA) acids, folic acid (FA) and the amino acid L-methionine (L-Met)), and its implication on the process of placentation, using human first trimester human EVTs (HTR-8/SVneo cell line). Moreover, we intended to develop an experimental cellular model of EVTs insufficiency and to revert this insufficiency, by improving the nutritional status of the cells.

Our results led us to conclude that in the HTR-8/SVneo cell line: a) GLU uptake seem to be mediated by the facilitative glucose transporter 1 (GLUT1), ARA uptake seem to be mediated by long-chain acyl-CoA synthetase 1 (ACSL1) and FA uptake seem to be mediated by proton-coupled folate transporter (PCFT); b) long-term (24h) exposure to xanthohumol (XN) interferes with placentation-related processes, as it decreases cell viability, culture growth,

proliferation, migration and increases apoptosis rates; c) the inhibitory effect of XN upon placentation-related processes is dependent on the cellular levels of GLU and ARA, as XN inhibits the cellular uptake of these nutrients and its effects on placentation-related processes were reverted by increasing the extracellular levels of either GLU or ARA; and d) ethanol (EtOH) and metformin (METF) inhibit GLU and FA cellular uptake, and interfere with placentation-related processes. In relation to the mechanism of action of these xenobiotics, the following intracellular signaling pathways were found to be implicated in the observed effects: a) the mammalian target of rapamycin (mTOR), c-Jun-N-terminal kinase (JNK) and tyrosine kinases (TK) appear to mediate the effect of XN upon ARA and GLU uptake; b) mTOR, JNK and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) appear to be involved in the effect of EtOH upon FA uptake and in the effect of METF upon cell viability; and c) mTOR and JNK appear to be involved in the effect of EtOH upon cell viability and GLU uptake.

In conclusion, exposure to different xenobiotics during pregnancy may affect the transport of essential nutrients to the placenta, and this may have a deleterious effect upon the placentation process, with detrimental consequences to fetal growth and development.

Keywords: apoptosis; arachidonic acid; extravillous trophoblast cells; folic acid; glucose; placenta; placentation; cell proliferation; cell migration; nutrient uptake; transmembrane transport; xanthohumol; xenobiotics.

RESUMO

A placenta humana constitui a principal ligação entre a mãe e o feto, sendo responsável pela troca materno-fetal de gases e nutrientes. Serve ainda de órgão endócrino, sendo responsável pela produção de hormonas associadas a gravidez, bem como de factores de crescimento e proteção do feto em desenvolvimento relativamente a resposta imunitária materna. A placentação – formação e desenvolvimento da placenta – é um processo contínuo e altamente regulado que se inicia imediatamente após fertilização e termina apenas aquando do parto. As células trofoblásticas extravilositárias (TEV) são células chave envolvidas no processo de placentação. Estas são células trofoblásticas com fenótipo invasivo e proliferativo; realizam a ancoragem das árvores vilositárias na cavidade intrauterina e regulam ativamente a remodelação das artérias espirais maternas, processo este que termina aquando do estabelecimento do fluxo sanguíneo utero-placentário.

O nosso principal objetivo foi investigar o impacto de xenobióticos no transporte materno-fetal de nutrientes essenciais ao crescimento e desenvolvimento placentário e fetal, mais especificamente a glicose (GLI), os ácidos gordos polinsaturados de cadeia longa ácido (araquidónico [AA] e ácido docosahexaenóico [ADH]), o ácido fólico (AF) e o aminoácido L-metionina (L-Met), bem como as suas implicações no processo de placentação. Para tal, recorremos a uma linha cellular de TEVs humanas de primeiro-trimestre de gravidez (linha celular HTR-8/SVneo). Adicionalmente, foi também nosso objectivo desenvolver um modelo celular experimental de TEVs insuficientes, bem como proceder à reversão desta insuficiência celular melhorando o seu estado nutricional.

Os nossos resultados levaram-nos a concluir que na linha celular HTR-8/SVneo: a) a captação de GLI parece ser mediada por transportadores

facilitativos de glucose 1 (GLUT1), a captação de AA parece ser mediada pela sintétase de acil-CoA de cadeia longa (ACSL) 1 e a captação de AF parece ser mediada pelo transportador de folatos acoplado a protões (PCFT); b) a exposição prolongada (24h) ao xantohumol (XN) interfere com processos relacionados com a placentação, dado que reduz a vibilidade, crescimento da cultura, proliferação e migração celulares e aumenta a taxa de apoptose celular; c) o efeito inibitório do XN relativamente a processos relacionados com a placentação é dependente de níveis celulares adequados de GLI e AA, dado que o XN inibe a captação celular destes nutrientes e os seus efeitos inibitórios nos processos relacionados com a placentação foram revertidos com o aumento das concentrações extracelulares de GLI ou AA; e d) o etanol (EtOH) e a metformina (METF) inibem a captação celular de GLI e AF, e interferem com processos relacionados com a placentação. Relativamente aos mecanismos de acção inerentes aos efeitos destes xenobióticos, as seguintes vias de sinalização intracelulares parecem estar envolvidas: a) o *mammalian target of rapamycin* (mTOR), cínase c-Jun-N-terminal (JNK) e cínases em Tirosina (TK), em relação ao efeito do XN na captação de GLI e AA; b) as vias mTOR, JNK e cínase do fosfatidilinositol-4,5-bisfosfato (PI3K), em relação ao efeito do EtOH na captação de AF e ainda ao efeito da METF viabilidade celular; e c) as vias mTOR e JNK, em relação ao efeito do EtOH na viabilidade celular e captação de GLI.

Em conclusão, os nossos resultados sugerem que a exposição a determinados xenobióticos durante a gravidez poderá afectar a captação de nutrientes essenciais à placenta, que pode decorrer em efeitos deletérios ao processo de placentação, com consequências prejudiciais ao crescimento e desenvolvimento fetais.

Palavras-chave: ácido araquidónico; ácido fólico; apoptose; captação de nutrientes; células trofoblásticas extravilositárias; glicose; migração celular; placenta; placentação; proliferação celular; transporte transmembranar; xantohumol; xenobióticos.

LIST OF ABBREVIATIONS

ACA	acetaldehyde
ACSL	long-chain acyl-CoA synthetases
ADH	alcohol dehydrogenase
AKT	protein kinase B
AMPH	amphetamine
AMPK	AMP activated protein kinase
ARA	arachidonic acid
ATP	adenosine-5'-triphosphate
BM	basal membrane
CAF	caffeine
CAT	cationic amino acid transporters
CC	cell columns
CK	cytokeratin
CM	cell membrane
COCA	cocaine
CYP2E1	cytochrome P450 2E1
CYT B	cytochalasin B
CTB	cytotrophoblast
DG	2-deoxy-D-glucose
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
4E-BP	4E-binding proteins
EGCG	epigallocatechin-3-gallate
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
EtOH	ethanol
evCTB	extravillous cytotrophoblasts
EVT	extravillous trophoblast
FA	folic acid
FABP	fatty acid binding proteins
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorders
FAT/CD36	fatty acid translocase
FATP	fatty acid transport proteins
FGR	fetal growth restriction
FLUOX	fluoxetine
FR	folate receptor
GLU	glucose
GLUT	facilitative glucose transporters
GSV	GLUT-storage vesicles
hCG	human chorionic gonadotropin
IL	interleukin
IRS	insulin receptors substrate proteins
JAK/STAT	janus kinases/signal transducers and activators of transcription
JNK	c-Jun-N-terminal kinase

L-Ala	L-Alanine
LA	linoleic acid
LAT	L-type amino acid transporter
LC-PUFAs	long-chain polyunsaturated fatty acids
LGA	large-for-gestational age
γ-LNA	γ-linolenic acid
L-Lys	L-Lysine
L-Met	L-Methionine
L-Ser	L-Serine
MAPK	mitogen-activated protein kinases
MCT	monocarboxylate transporter
MDMA	ecstasy, 3,4-Methylenedioxymethamphetamine
METF	metformin hydrochloride, 1,1-dimethylbiguanide hydrochloride
5-MTHF	5-methyltetrahydrofolate
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NICO	nicotine
NTB cells	human cytotrophoblasts obtained from normal pregnancies
NTDs	neural tube defects
OA	oleic acid
PA	palmitic acid
PCFT	proton-coupled folate transporter
pFABPpm	placental plasma membrane fatty acid binding protein
PHT	phloretin
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PPAR	peroxisome proliferator-activated receptor
Px	peroxisome
QUE	quercetin
RAPA	rapamycin
RESV	resveratrol
RFC	reduced folate carrier
RG	rosiglitazone
RNA	ribonucleic acid
ROS	reactive oxygen species
S6K	ribosomal S6 kinases
SGLT	sodium-dependent glucose co-transporters
SNAT	sodium-coupled neutral amino acid transporter
STB	syncytiotrophoblast
TB	trophoblast
TK	tyrosine kinases
TEO	theophylline
TNF-α	tumor necrosis factor-alpha
TSC	tuberous sclerosis complex
vCTB	villous cytotrophoblasts
VEGF	vascular endothelial growth factor
XN	xanthohumol

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INTRODUCTION

1. HUMAN PLACENTA

The human placenta is the main interface between the maternal and fetal blood circulations [1]. It is a transient organ of fetal origin, which performs multiple functions required for the growth and development of the fetus, namely: a) anchoring the conceptus to the uterine wall and prevention of its rejection by the maternal immune system [1, 2]; b) mediation of the exchange of nutrients, respiratory gases, water and waste metabolites between maternal and fetal blood [1]; and c) production of hormones which can be divided in different functional classes according to , such as steroids (e. g. progesterone, oestradiol, glucocorticoids), polypeptides (e. g. prolactin, human placental lactogen, human placental growth hormone), glycoproteins (e. g. human chorionic gonadotropin (hCG)), adipokine (e. g. leptin, adiponectin, resistin), eicosanoids (e. g. prostaglandins), cytokines (e. g. interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α)) and growth factors (e. g. placenta growth factor, vascular endothelial growth factor (VEGF)), that can be secreted into both the fetal and maternal circulations [1-4].

1.1. Placental development – the placentation process

Placentation – formation and growth of the placenta - is a continuous and highly regulated process that begins immediately after fertilization and ends only after delivery [5]. An adequate placentation and a consequent satisfactory nutrient supply to the fetus are crucial factors for fetal development and growth and for a good pregnancy outcome. Such critical

importance is evident as fetal growth restriction (FGR) and preeclampsia are characterized by impaired uterine blood flow and placental development causing reduced fetal nutrient uptake and fetal hypoxia [5].

Early after fertilization, the process of blastocyst implantation begins along with the differentiation of trophoblastic stem cells into two cell lineages: a) villous cytotrophoblasts (vCTBs) which fuse to form the multinucleated syncytiotrophoblast (STB), that ensure placental endocrine, protective and transport functions, and b) specialized extravillous trophoblasts (EVTs), which are the main participants in the process of placentation (Fig. 1.). EVTs are fully specialized trophoblasts exhibiting an invasive and proliferative phenotype; they invade the myometrium and uterine vasculature, perform the anchorage of the chorionic villi into the uterine wall and actively regulate uterine spiral arteries remodeling, a process that ends up with the establishment of the utero-placental blood flow [6].

1.2. Cell models of placentation

The HTR-8/SVneo cell line was established 3 decades ago by transfecting HTR-8 cells with a plasmid containing the gene for the simian virus 40 (SV40) large T antigen (Tag). Thus, similarly to choriocarcinoma cells, HTR-8/SVneo cells display an unlimited lifespan in culture. Primary cultures of HTR-8 cells were first obtained by the outgrowth of cells from tissue explants of normal first trimester villi. These primary cells share features with invasive trophoblasts such as expression of cytokeratin (CK) 18 and some EVT-specific integrins [7], but they present a limited lifespan in culture. As such, the transformed HTR-8/SVneo cell line was established as a human first trimester EVTs cellular model.

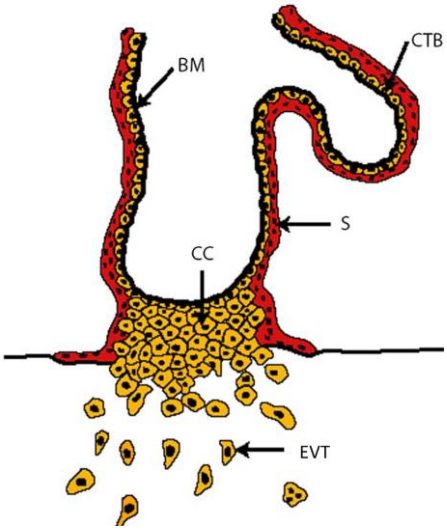


Fig. 1. Formation and differentiation of extravillous trophoblasts.

During anchorage of chorionic villi at the uterine BM, the mononucleated CTB undergo either: a) fusion and biochemical differentiation to originate the multinucleated syncytiotrophoblast/syncytium; or b) originate proliferative CC constituted of proliferative and invasive EVTs. BM: basal membrane; CC: cell columns; CTB: cytotrophoblast; EVT: extravillous trophoblasts; S: syncytium. *Adapted from Pollheimer, J. and M. Knofler, Signalling pathways regulating the invasive differentiation of human trophoblasts: a review. Placenta, 2005. 26 Suppl A: p. S21-30.*

HTR-8/SVneo cells possess trophoblastic (secretion of hCG) and epithelial (expression of CK 8 and 18) characteristics and they show proliferative and invasive characteristics of first-trimester EVTs [7]. Therefore, HTR-8/SVneo cells are an accepted *in vitro* cell model system for the study of first-trimester EVTs characteristics [7]. Interestingly, a recent study showed that the HTR-8/SVneo cell line contains two distinct populations of cells, namely trophoblast and stromal/mesenchymal cells. HTR-8/SVneo cell cultures contained few clusters of the

trophoblast/epithelial marker CK7 positive cells and the mesenchymal marker vimentin positive cells. It is claimed that at the tips of the anchoring villi, evCTB undergo a transition from epithelial-to-mesenchymal phenotype, thus these cells loose epithelial markers and acquire mesenchymal markers when they start to invade and migrate into the maternal endometrium and spiral arteries [8]. As such, the fact that the HTR-8/SVneo cell line presents stromal/mesenchymal populations of cells might reflect what undergoes *in vivo* during the placentation process.

There is an assortment of additional EVT cell models, such as SGHPL-4, SGHPL-5 and HIPEC 65 cells, that have been developed from first trimester placentas after transfection with SV40 large T antigen and express cytokeratin 7 and HLA-G and retain cellular functions of *in vivo* EVT (reviewed by [9]). However, these cell lines retain a senescence mechanism being commonly used only until passage 25, and are suggested to show a fibroblastoid phenotype instead of the expected EVT epithelial phenotype (reviewed by [9]). Additionally, other existing EVT cell lines (such as HT-116 or SWAN 71) have been hardly used for *in vitro* EVT studies, as their lifespan in culture is also limited (reviewed by [9, 10]). So, HTR-8/SVneo cells appears to be the most accepted *in vitro* model system for the study of first-trimester EVTs characteristics, including cell fusion, proliferation, migration and invasion. As such, our studies will focus on this cell line.

It is worth of note that, given the important role of EVTs in placentation and knowing that FGR is associated with insufficient EVTs, a cell model of insufficient EVTs, as observed in FGR, would be crucial in order to comprehend the mechanism underlying the normal and abnormal process of placentation. Unfortunately, such a model is presently lacking.

2. PLACENTAL TRANSPORT OF NUTRIENTS

The placenta plays a major role in fetal nutrition, growth and development by transplacental allocation of nutrients from the maternal to fetal circulation [11-13]. Furthermore, the placenta itself requires nutrients for its own adequate growth and development, and since the majority of essential nutrients are not synthesized by the fetus or placenta, at least in sufficient amounts, placental nutrient transport from the maternal circulation is crucial. It is widely accepted that an impaired capacity of placenta to metabolize and supply nutrients to the fetal circulation may be a critical factor for the establishment of pregnancy-related pathologies such as preeclampsia and FGR [14-16].

The subject of this study was to characterize transport of the nutrients glucose, the long-chain polyunsaturated fatty acids (LC-PUFAs) arachidonic (ARA) and docosahexaenoic (DHA) acids, folic acid (FA) and the amino acid L-methionine (L-Met), to investigate the impact of xenobiotics upon these mechanisms and the implications of nutrient uptake modulation on the process of placentation, by using a first trimester human EVTs cell line (HTR-8/SVneo cells). So, in the following section we will describe the central principles of glucose, LC-PUFAs, FA and amino acids placental transport.

2.1. Glucose Transporters

Glucose is a major substrate for fetal and placental energy metabolism, and together with amino acids, it constitutes the primary stimuli for fetal secretion of the growth-promoting hormone insulin [17]. Gluconeogenesis in the feto-placental unit is minimal [18, 19]; therefore, allocation of glucose from the maternal circulation is critical for the process of placentation and for

normal fetal development and growth [11, 20]. Despite such importance, glucose homeostasis in first-trimester trophoblasts and the implications of this mechanism in the process of placentation are still largely unexplored.

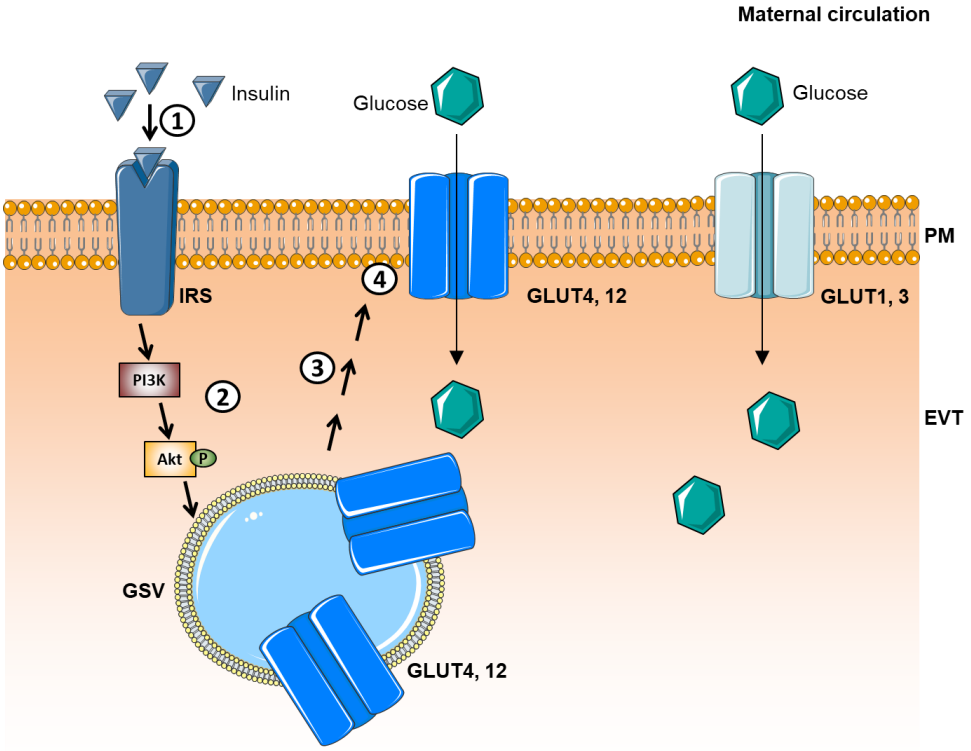


Fig. 2. Glucose transport in first trimester human trophoblasts.

Insulin-dependent GLUTs signaling: (1) insulin binds to its receptor leading to activation of IRS; (2) activation of PI3K/Akt signaling pathway; (3) trafficking of GSVs next to the plasma membrane; (4) tethering/docking and further fusion of GSVs containing GLUT4 or 12 with the plasma membrane. Insulin-independent GLUTs signaling, most probably GLUT1 and GLUT3. EVT: extravillous trophoblasts; IRS: insulin receptors substrate proteins; GLUT: glucose transporter; GSV: GLUT4 (and 12)-storage vesicle; PM: plasma membrane.

Placental transport of glucose was first characterized in syncytial microvillous and basal membranes to be sodium-independent, which excludes the involvement of the sodium-dependent glucose co-transporters (SGLTs, gene symbol SLC5A) (as reviewed elsewhere [11, 21]). On the other hand, studies have shown that membrane transporters are selective for D- over L-glucose and sensitive to inhibition by phloretin (PHT) and cytochalasin B (CYT B), pointing thus to the involvement of the facilitative sodium-independent sugar transporters [glucose transporter (GLUT) family, gene symbol SLC2A], a group comprising at least 14 isoforms (GLUT1–GLUT14) [19, 21, 22]. Among the 14 isoforms, GLUT1 is a ubiquitous isoform, expressed in almost all tissues and it is regarded as the constitutive form of the glucose transporter in the placenta from implantation to term [17, 20-22]. Besides GLUT1, other glucose transporters such as GLUT3 and the insulin-sensitive GLUT4 and 12, are found in first trimester STB, and play an important role earlier in gestation [11, 22]. The insulin-sensitive GLUT4, and hypothetically also GLUT12, are classically described in insulin-responsive tissues such as skeletal muscle and adipose tissue to be retained in intracellular storage vesicles termed GLUT-storage vesicles (GSVs) (reviewed in [23]). Briefly, and as can be observed in Fig. 2, insulin binds to its receptor leading to activation of the insulin receptor substrate (IRS) proteins. This activates the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway and subsequent translocation of GLUT4/12 from GSVs to the plasma membrane occurs, by tethering/docking of GSVs and further fusion with the plasma membrane [23]. GLUT4/12 can be further retrieved from the plasma membrane by endocytosis [23]. Regarding GLUT4, it is known that: 1) in term TBs, the majority of GLUT4 are localized in low-density microsomes and its distribution was not altered by insulin treatment [11, 24]; 2) GLUT4 has not been identified in membranes from primary STB or CTB cells [11, 24]; and 3) GLUT4 levels of expression in JAR choriocarcinoma cells were extremely low compared with those of GLUT1 and GLUT3,. Overall,

these results suggest that unlike the importance of GLUT4 in first-trimester TBs, GLUT4 does not contribute significantly to cellular glucose uptake in term TBs [11, 25]. Concerning GLUT12, this isoform is expressed in first-trimester STB and vCTB and it is absent at term in these cells, suggesting a vital function in early gestation [11, 26]. As for GLUT3, it has been described to be expressed in CTB and SCT in early gestation and decreases thereafter, being mainly expressed in endothelial cells at term [21, 27]. Curiously, despite the recognized deficits in fetal plasma glucose concentrations in FGR fetuses (reviewed in [21]), GLUT1 expression in placentas from term or preterm FGR appears to be unaffected in this condition (reviewed in [21]).

2.2. LC-PUFAs Transporters

The developing placenta and the fetus require the nutritionally essential fatty acids linoleic (LA; 18:2n-6) and γ-linolenic (γ-LNA; 18:3n-3) acids, which are obtained predominantly from vegetable oils, and their respective long-chain polyunsaturated fatty acids (LC-PUFAs) derivatives, arachidonic (ARA; 20:4n-6) and docosahexaenoic acids (DHA; 22:6n-3) [28, 29]. As reviewed elsewhere [29], ARA is a major precursor for the synthesis of eicosanoids such as prostaglandins, thromboxanes and leukotrienes, which are important for the development of fetal nervous, visual, immune and vascular systems [29, 30] and DHA is crucial for the development of the fetal neurovisual system and is highly accumulated in the brain and retina [29, 30]. As such, a deficiency of both ARA and DHA during intrauterine life is associated with cognitive, behavioral and visual abnormalities later in life [31]. In agreement with this, several studies suggest that maternal LC-PUFAs supplementation during pregnancy improves neurodevelopmental functions of the infants (reviewed in [32]) and, at the same time, reduces the risk of

preterm delivery [33]. In fact, studies recommend DHA supplementation when less than the desirable maternal plasma levels are achieved through dietary habits [34]. Worth of note, alterations in placental LC-PUFAs transport may occur in pregnancies complicated by FGR, and fetal-to-maternal blood ratio of ARA and DHA was found to be lower in FGR pregnancies compared with normal pregnancies [35].

LC-PUFAs transported across the human placenta are mainly delivered from triglyceride-rich lipoproteins (from which they must be released by the action of placental lipases) and from fatty acids bound to albumin [35]. Transfer of fatty acids from maternal to fetal circulation may be driven by simple passive diffusion [36]. However, in tissues with high fatty acid demand, uptake of fatty acids by simple diffusion may be insufficient to meet minimum requirements [36]. Although LC-PUFAs cannot be synthesized by the fetus, they are present at higher levels in fetal circulation, in relation to maternal circulation [36]. This is consistent with an active and selective placental transfer of these fatty acids in favor of the fetal compartment – a process described as "**biomagnification**" [36]. At the STB transporting epithelium level, both passive diffusion and protein-mediated transport seem to be involved in cellular uptake of LC-PUFAs from the maternal circulation, namely [30, 37] (Fig. 3.):

a) placental plasma membrane fatty acid-binding protein (pFABPpm), an unidirectional placenta-specific transporter;

b) ATP-dependent fatty acid transport proteins (FATPs); and

c) fatty acid translocase (FAT/CD36), a glycoprotein receptor that allows bidirectional and non-selective transport of fatty acids

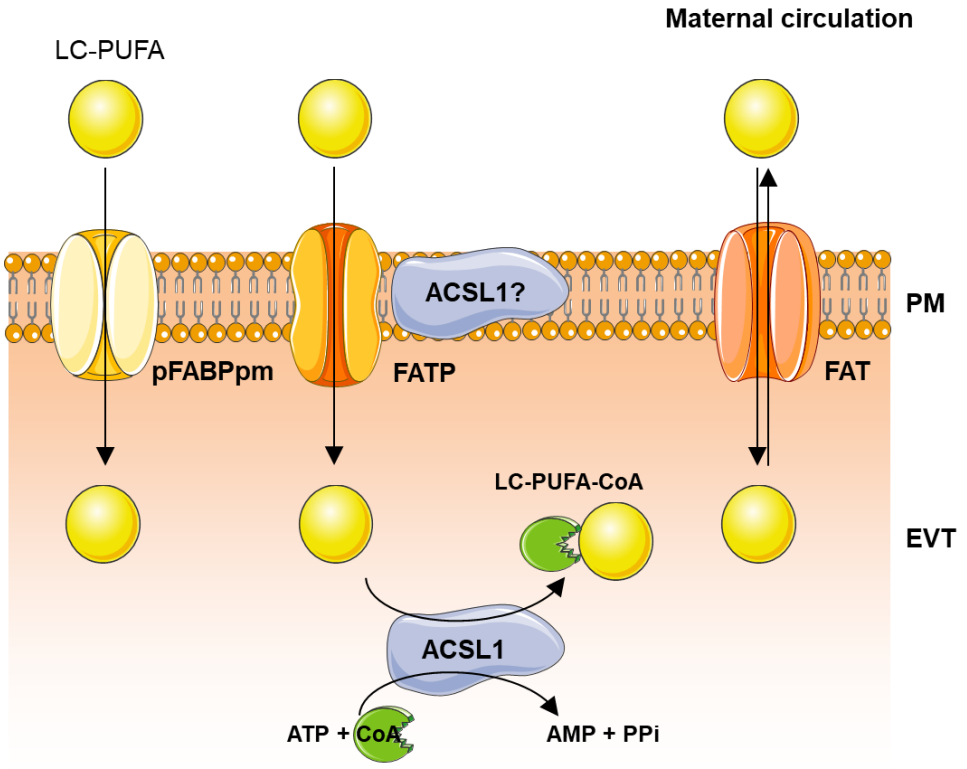


Fig. 3. LC-PUFAs transport in first trimester human trophoblasts.

LC-PUFAs uptake from maternal circulation into the EVTs is mediated by pFABPpm, FAT and FATP. It is unknown whether FATP has ACSL1 activity or ACSL1 is active as a LC-PUFAs transporter in PM. ACSL1 present in the cytoplasm performs the activation of LC-PUFAs in a two-step reaction : 1) an acyl-AMP intermediate is formed from ATP; and 2) AMP is then exchanged with CoA to produce the activated acyl-CoA. ACSL1: long-chain acyl-CoA synthetases; AMP: adenosine monophosphate; ATP: adenosine triphosphate; CoA: coenzyme A; EVT: extravillous trophoblasts; FAT: fatty acid translocase; FATP: ATP-dependent fatty acid transport proteins; pFABPpm: plasma membrane fatty acid-binding protein; LC-PUFA: long-chain polyunsaturated fatty acids; LC-PUFA-CoA: long-chain polyunsaturated fatty acid-coenzyme A; PPi: pyrophosphate; PM: plasma membrane.

A still ongoing debate about FATP is whether they act solely as transport proteins and/or acts in concert with intracellular long-chain acyl-CoA synthetases (ACSLs) or if FATP itself also harbors ACSL activity [38]. ACSLs alone also contribute to the process of LC-PUFAs uptake, as this group of cytosolic enzymes converts LC-PUFAs into acyl-CoA derivatives for further esterification or β-oxidation, thus preventing the efflux of the incorporated fatty acids [37, 39, 40]. Hence, ACSLs allows the L-PUFAs uptake to be unidirectional and trapping them inside the cell [37, 39, 40]. The activation of LC-PUFAs by ACSLs requires a two-step reaction (Fig. 3.): 1) an acyl-AMP intermediate is formed from ATP; and 2) AMP is then exchanged with CoA to produce the activated acyl-CoA [41]. Overall, the precise mechanisms by which these transport proteins facilitate the allocation of LC-PUFAs through the placenta are still a matter of speculation.

2.3. FA Transporters

Folic acid (pteroylglutamate; FA) is the parent structure and oxidized form of a large family of B_9 family of water-soluble vitamin coenzymes known as folates [42-44]. These molecules facilitate the transfer of one-carbon units in reactions leading to the synthesis of methionine, thymidine, purine and pyrimidine precursors of nucleic acids, the metabolism of certain amino acids, and the initiation of protein synthesis in mitochondria [42-44]. FA is of major importance for normal fetal development, as evidenced by the well-established association between maternal folate deficiency and low birth weight and increased risk of spontaneous abortion and of neural tube defects (NTDs) (e.g. spina bifida and anencephaly) [42-45]. FA importance in pregnancy is also demonstrated by the accumulating evidence that

supplementation with this vitamin during the periconceptional period can reduce the incidence of low birth weight newborns and NTDs [42-45].

The human placenta expresses:

a) the reduced folate carrier (RFC1), a folate:organic phosphate exchanger that utilizes the transmembrane organic phosphate gradient to mediate the uptake of folates and has a maximal activity at physiological pH [46-49];

b) the folate receptor α (FRα) [46, 49, 50] and folate receptor β (FRβ) [49, 51], high-affinity folate-binding proteins embedded in the membrane by a glycosylphosphoinositol anchor that mediate the unidirectional uptake of folates at neutral to mildly acidic pH; and

c) the more recently described proton-coupled folate transporter (PCFT), a high affinity folate:H^+ symporter, with an optimal activity at acidic pH (5.5–6.0) [49, 52, 53]. PCFT is known to co-localize with FRα [49, 52].

All of these transporters act coordinately to ensure the vectorial transfer of folates from maternal to fetal circulation [49, 52, 53]. However, it is widely accepted that PCFT and FRα appear to have a major role in the process of FA transport from maternal to fetal circulation. In fact, a model for folates transport across the placenta has been proposed (Fig. 4.), in which: 1) folates bind to FRα; 2) co-localization of this transporter with PCFT allows the internalization of both into an endosome; 3) during cytoplasmic transit, this endosome is acidified (pH 6.0–6.5) due to an influx of protons through a vacuolar proton ATPase, promoting the dissociation of folates from FRα and establishing a favorable H^+ gradient that allows PCFT-mediated folate efflux

into the cytoplasm; and 4) FRα and PCFT are then recycled back to the membrane surface [49, 53, 54].

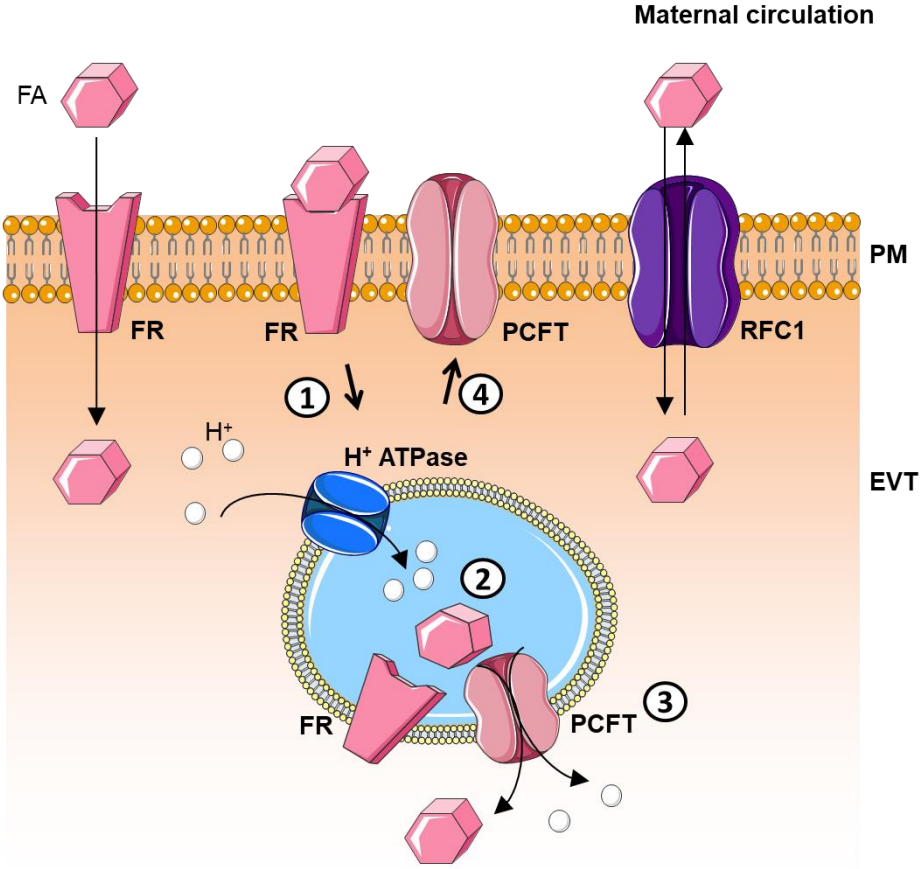


Fig. 4. Folic acid transport in first trimester human trophoblasts.

Model of internalization of PCFT and FRα: (1) folates bind to FRα and FRα with PCFT are internalized into endosomes; (2) acidification of the endosome by vacuolar H^+-ATPase; (3) folates are dissociated from FRα and are transported to cytoplasm by PCFT-mediated efflux favored by a H^+ gradient; (4) FRα and PCFT are recycled back to the cell membrane surface. EVT: extravillous trophoblasts; FA: folic acid; FR: folate receptor; H^+ ATPase: proton ATPase; PCFT: proton-coupled folate transporter; PM: plasma membrane; RFC1: reduced folate carrier.

Studies by our team showed that placental uptake of FA is PCFT-mediated and downregulated by chronic hyperglycemia, some anti-hypertensive drugs, drugs of abuse and ethanol [53], and is differently modulated by polyphenols and methylxanthines [55]. Interestingly enough, and contrary to the expected, an increase in placental uptake of folates has been demonstrated in FGR, suggesting that the placenta exhibited “a compensation for the weakness effect" [14].

2.4. L-Methionine Transporters

Amino acids are used as building blocks for fetal protein synthesis, and are important energy substrates for both the fetus and the placenta [17]. Moreover, they are biosynthetic precursors of purines, pyrimidines, some neurotransmitters, nitric oxide, glutathione, polyamines and haem [56].

L-Met is a nutritionally essential large neutral amino acid that plays a critical role in one-carbon metabolism. After conversion to S-adenosylmethionine, this amino acid provides the methyl groups required for the methylation of DNA, RNA, proteins, biogenic amines and phospholipids. Notably, the importance of L-Met during pregnancy is observed by the higher occurrence of NTDs in women with low dietary intake of L-Met, and FGR in animal models of L-Met intake restriction during pregnancy [57]. Interestingly enough, in placentas obtained from FGR newborns, the activity and expression of L-Met transport systems (A, L and y+L, described below) were shown to be reduced [32].

The concentration of most amino acids, including L-Met, at the placental level is normally higher than that found in both fetal and maternal circulation - a process that can also be described as **“biomagnification”** (as

above described for LC-PUFAs) [56], suggesting that their uptake into the placenta occurs mainly by active processes [58], whereas their transport into the fetal blood is mainly passive.

Based on the functional characteristics of amino acid transport systems, such as substrate specificity, inhibition by L-Met and placental-specific location, four different transporters have been identified in the human STB as capable of transporting L-Met (Fig. 5.):

a) the sodium-dependent system A, which mediates the uptake of neutral amino acids (both essential- and non-essential) with short and unbranched side chains, mostly L-Ala, Gly, L-Ser, L-Met and L-Gln. The most commonly expressed isoforms are the sodium-coupled neutral amino acids transporter 1 (SNAT1), SNAT2 and SNAT4 [56, 58, 59];

b) the sodium-independent system L, which mediates the uptake of many neutral (eg. L-Met), branched-chain (eg. L-Leu) and aromatic (eg. L-Phe and L-Tyr) amino acids, and includes isoforms with polarized expression in the STB, such as L-type amino acid transporter (LAT) 1-4 [56, 59]; and

c) the two cationic amino acid transport systems y^+L and b^{0+}[56, 59-61].

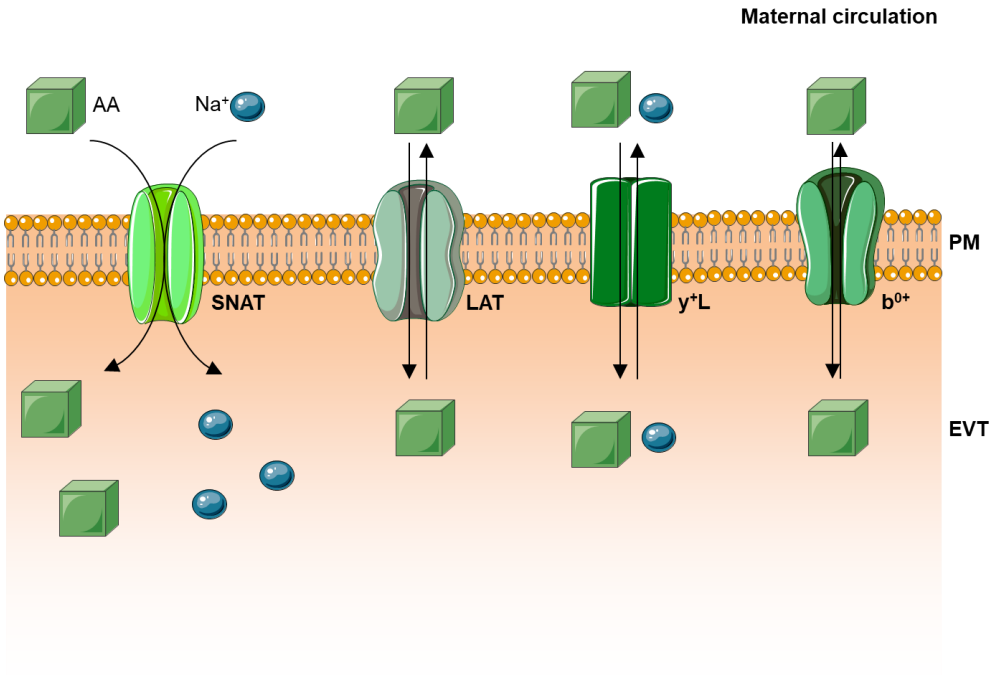


Fig. 5. Amino acid transport in first trimester human trophoblasts.

AA uptake from maternal circulation into the EVTs is mediated by SNAT, LAT, y^+L and b^{0+}. AA: amino acid; EVT: extravillous trophoblasts; LAT: L-type amino acid transporter, PM: plasma membrane; SNAT: sodium-coupled neutral amino acids transporter.

3. XENOBIOTICS AND PREGNANCY

Pregnant women are frequently exposed to several xenobiotics due to lifestyle factors such as diet, medication, smoking, alcohol consumption or drug abuse.

It is widely accepted that the consumption of drugs of abuse, such as cocaine (COCA), amphetamines (AMPH), *ecstasy* (3,4-methylenedioxymethamphetamine; MDMA), nicotine (NICO) and alcohol (ethanol, EtOH) exert deleterious effects on the fetus [62].

Besides nutrients, diet provides bioactive compounds such as polyphenols and methylxanthines, which are present in fruits and vegetables and in both alcoholic (red wine, beer) and non-alcoholic (tea) beverages.

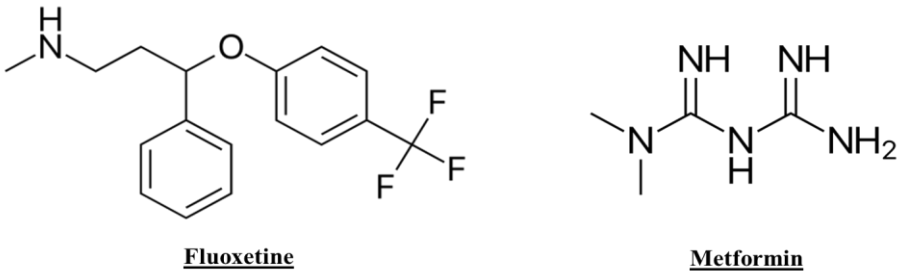
Moreover, pregnant women often take medication for the treatment of various pregestational or gestational conditions such as epilepsy, hypertension, depression and type 2 and gestational diabetes.

These families of xenobiotics are known to readily cross the placenta [63, 64], and thus the study of their effects upon the placenta and the fetus is of major importance. However, the effect of these xenobiotics upon nutrient uptake by first trimester human EVTs and upon the placentation process is still largely unexplored.

3.1. Therapeutic drugs

Fluoxetine (FLUOX) (Fig. 6.) is an antidepressant drug that belongs to the selective serotonin reuptake inhibitors (SSRI) class. During pregnancy and in the immediate postnatal period, 14-23% of women present a depressive disorder [65, 66], and it is estimated that 2–3% of pregnant women receive SSRI antidepressant treatment [65].

Metformin (METF) (1,1-dimethylbuguanide hydrochloride) (Fig. 6.) is a biguanide agent that reduces hyperglycaemia by suppressing hepatic glucose output (hepatic gluconeogenesis), increasing insulin sensitivity and improving peripheral glucose uptake [67, 68]. METF is widely used as first-line treatment in type 2 diabetes and polycystic ovary syndrome (PCOS) [69] and is becoming increasingly accepted as an alternative to insulin during pregnancy, for the management of type 2 and gestational diabetes and PCOS [70-73]. It is worth of note that METF freely crosses the placenta, thus exposing the fetus to concentrations approaching those in the maternal circulation [69, 74]. However, the effects of METF on the placenta and its long-term effect on fetal physiology have not been well elucidated [72, 73].



Fluoxetine Metformin

Fig. 6. Molecular backbone structures of the therapeutic drugs Fluoxetine and Metformin.

3.2. Drugs of abuse

It is widely accepted that the consumption of drugs of abuse, including alcohol (ethanol; EtOH), exerts deleterious effects on the fetus [62]. The

placenta and the fetus become frequently exposed to drugs of abuse because of maternal use of these drugs. For instance, in an US study, nearly 25% of pregnant women had a positive toxicology screen for any illicit drug and nearly 10% had a positive screen for COCA, AMPH, MDMA, and/or opiates (Fig. 7.) [75]. In relation to EtOH (Fig. 7.), it is one of the most frequently used drug worldwide [76], and although studies suggest that most women avoid alcohol during pregnancy, in the U.S. population still 10% of pregnant women report alcohol use [77]. EtOH is widely accepted as a potent teratogen in humans [78, 79] and is one of the most consumed drug of abuse during pregnancy [77].

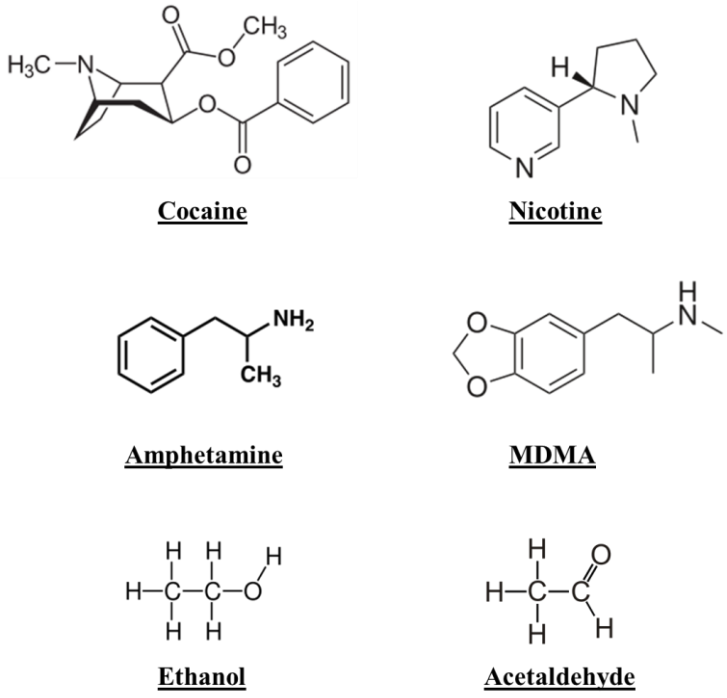


Fig. 7. Molecular backbone structures of the drugs of abuse Cocaine, Nicotine, Amphetamine, MDMA, Ethanol and Acetaldehyde.

Acetaldehyde (ACA) (Fig. 7.) is the principal metabolite of hepatic EtOH catabolism (Fig. 8.) and is a highly reactive molecule that can form adducts with biological molecules, thus inactivating them. As such, part of the toxic effects associated with the ingestion of EtOH is related to ACA formation [80]. It is worth to note that EtOH is naturally present in the blood at low concentration (0.39 ± 0.45 μg/mL) due to carbohydrate fermentation by the bacterial gastrointestinal flora after which it reaches blood circulation by simple diffusion from the gastrointestinal tract [80, 81]. Also, there is a still on-going debate on the possibility of ethanol formation inside human tissues, thus accounting to an ethanol endogenous production [81]. However, from the point of view of this study, we will consider this compound as a drug of abuse and we will focus on concentration levels superior to the physiologically observed.

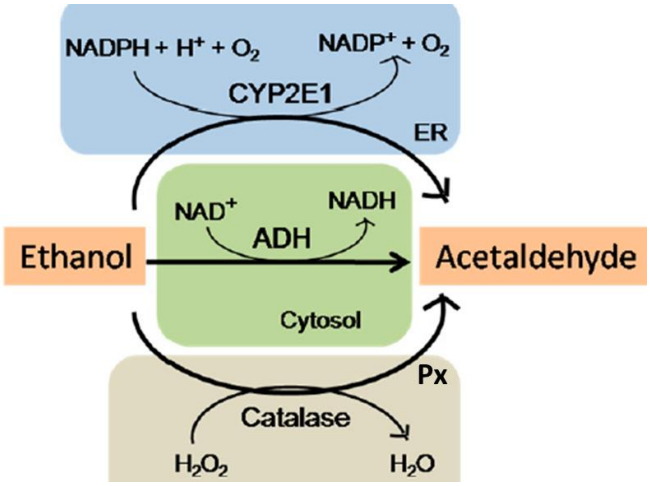


Fig. 8. Pathways of ethanol metabolism.

Ethanol is oxidized mainly by cytosolic ADH to acetaldehyde. Another major pathway of ethanol metabolism includes its oxidation in microsomes by CYP2E1 and requires NADPH instead of NAD^+. ADH: alcohol dehydrogenase; CYP2E1: cytochrome P450 2E1; ER: endoplasmic reticulum; NAD^+: nicotinamide adenine dinucleotide; NADPH: nicotinamide adenine dinucleotide phosphate; Px: peroxisome. *Adapted from Ratna, A. and P. Mandrekar, Alcohol and Cancer: Mechanisms and Therapies. Biomolecules, 2017. 7(3).*

Alcohol consumption during pregnancy can lead to brain, craniofacial, cardiovascular and limb defects collectively termed Fetal Alcohol Spectrum Disorders (FASD) [77, 82]. Among children with FASD, a small population presents a specific set of anomalies (specific facial abnormalities, fetal growth retardation and significant impairments in neurodevelopment) known as Fetal Alcohol Syndrome (FAS) [78, 79, 83]. Furthermore, prenatal alcohol exposure can lead to other alcohol-related birth defects such as decreased immune function, attention problems and hearing impairment, but also spontaneous abortion [78].

Finally, maternal smoking and exposure to nicotine (NICO) during pregnancy may cause FGR [84, 85]. Approximately 14% of U.S. women continue to smoke after becoming pregnant [86]. Interestingly enough, NICO metabolism increases in pregnancy, which may contribute to the difficulty that pregnant women have in quitting smoking [86].

3.3. Dietary bioactive compounds

3.3.1. Polyphenols

In this work we also focused our attention in studying the effects of several polyphenols, which are secondary plant metabolites recognized for their powerful antioxidant properties [87, 88]. Polyphenols are present in several fruits and vegetables and in alcoholic (eg. red wine, beer) and non-alcoholic (eg. tea, fruit juices) beverages [87, 88]. Polyphenols are chemical compounds with more than one hydroxyl functional group (–OH) attached to an aromatic ring that underlies the distinctive physical, chemical, and biological properties of the particular class members. Polyphenols can be classified on two major distinguishable subgroups, flavonoids and non-

flavonoids, as can be observed in Fig. 9. [88, 89]. Flavonoids consist of 15 carbon atom compounds (C6–C3–C6) characterized by two benzene rings joined by a three carbon chain forming an oxygenated heterocycle [89]. The non-flavonoids comprise a more heterogenous group: phenolic acids; phenolic alcohols; stilbenes; chalcones; and other polyphenols [89]. Out of the polyphenols found in diet, 60% are represented by flavonoids and 30% by phenolic acids [87, 89].

Several lines of evidence emerged in the last two decades attributing health-protective effects to these bioactive substances. Indeed, polyphenols are known to have antioxidant, anti-proliferative and anti-inflammatory properties, and to be protective against cancer, cardiovascular and inflammatory diseases [90, 91]. In this work, we focused our study on the polyphenols epigallocatechin-3-gallate (EGCG), quercetin (QUE), resveratrol (RESV) and xanthohumol (XN) (Fig. 10.).

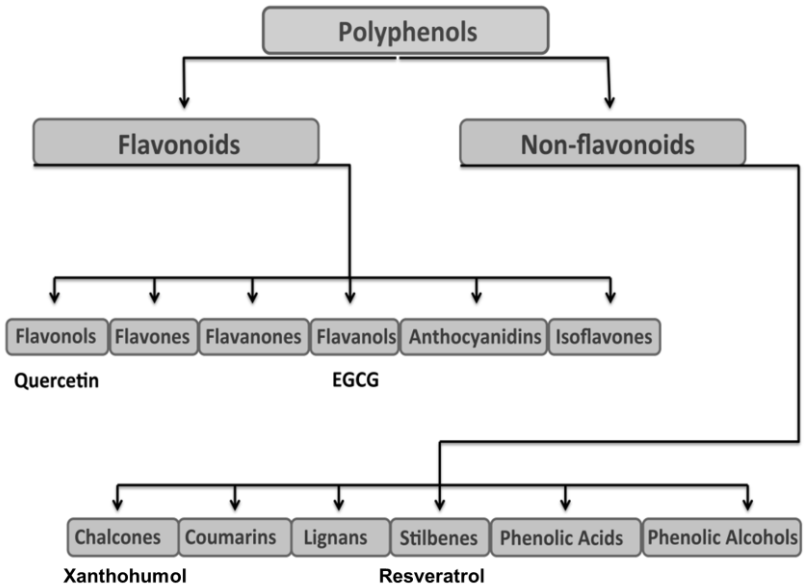


Fig. 9. Major sub-classes of flavonoid and non-flavonoid polyphenols.

Adapted from Teixeira, J., et al., Dietary Polyphenols and Mitochondrial Function: Role in Health and Disease. Curr Med Chem, 2017; 24: 1-27.

EGCG (epigallocatechin-3-gallate) is the ester of epigallocatechin and gallic acid, and is the most abundant type of catechin in tea (e.g. green and white tea) [92]. QUE (3,3′,4′,5,7-pentahydroxyflvanon) is categorized as a flavonol and is found in a variety of foods including apples, onions, berries, grapes, shallots, tea, and tomatoes, as well as in many seeds, nuts, flowers, barks, and leaves [93]. RESV (3, 4', 5-trihydroxystilbene) is a stilbenic phenolic compound, present mainly in grape and grape products, but also in lower amounts in peanuts, pistachios, some berries, tomato skin, and chocolate [91]. XN (3'-[3,3-dimethyl allyl]-2',4',4-trihydroxy-6'-methoxychalcone) – is a structurally simple prenylated chalcone that occurs in the hop plant and is used to add bitterness and flavor to beer [94].

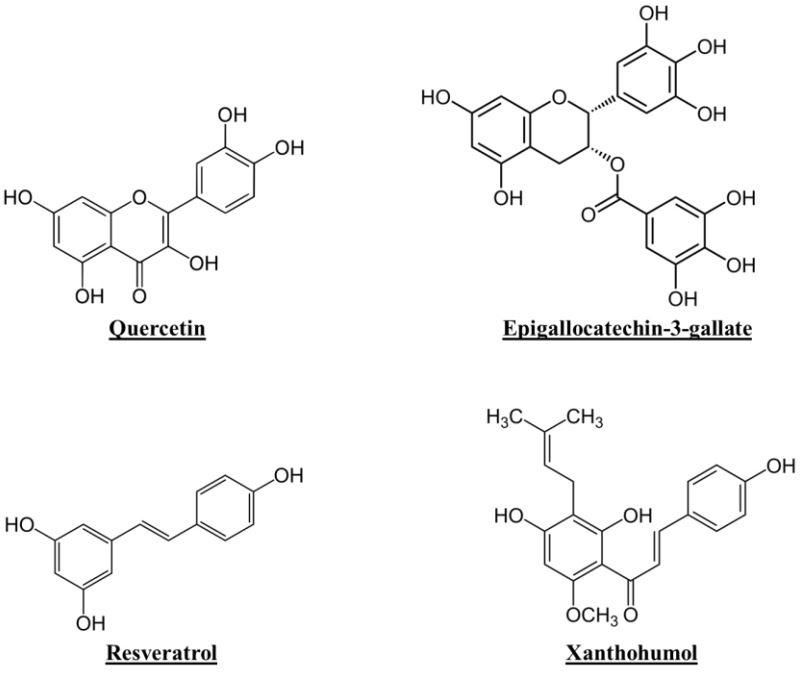


Fig. 10. Molecular backbone structures of the polyphenols Quercetin, Epigallocatechin-3-gallate, Resveratrol and Xanthohumol.

Worth to note that most polyphenols, except catechins, can exist in the diet as glycosides (such as glucoside, galactoside, rhamnoside, arabinoside, and rutinoside) and are considered too hydrophilic for absorption by passive diffusion in the small intestine, thus only aglycones or the resulting aglycones from hydrolyzation (deglycosylation) of the glycoside form were likely to be absorbed [95]. Interestingly enough, some studies point to the possibility that the glycoside form of the polyphenols might be actively transported across the enterocyte [95].

3.3.2. Methylxanthines

Methylxanthines such as caffeine (1,3,7-trimethylxanthine, CAF) and its demethylated metabolite theophylline (1,3-dimethylxanthine, TEO) rank as one of the topmost commonly consumed dietary ingredients throughout the world [96, 97] (Fig. 11.). Drinks containing CAF and TEO (e.g. coffee, tea, soft drinks) are often consumed by pregnant women; indeed, 75% of pregnant women in the US consume low to moderate amounts of CAF [98]. Both CAF and TEO readily pass the placenta barrier [64] and thus might affect the fetus, as the immature fetal liver enzyme CYP3A4 is unable to metabolize them [99]. Thus, their effect on the placenta and fetus should be explored. The FDA recommends that pregnant women should avoid the ingestion of CAF due to observed teratogenic effects (e.g. spontaneous abortion and FGR) [98, 100]. However, there is still some debate as to the CAF real detrimental effect to the fetus, mostly due to confounding associated maternal risk behavior factors, such as tobacco use [63, 101, 102]. For this reason, CAF present in coffee and commonly consumed by pregnant women, and its metabolite TEO, are two xenobiotics whose placental effects are worth of study.

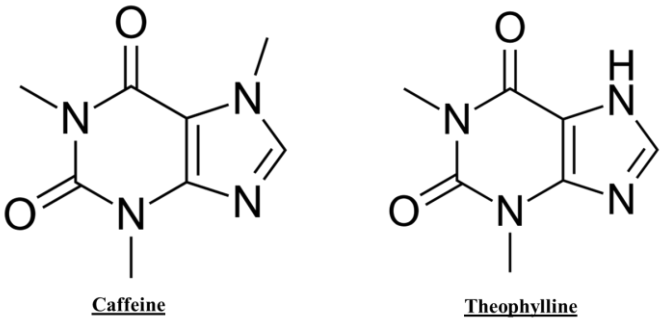


Fig. 11. Molecular backbone structures of the methylxanthines Caffeine and Theophylline.

4. THE PLACENTA AS A NUTRIENT SENSOR

The placenta responds to perturbations in the maternal environment (e.g. hypoxia, stress, obesity, diabetes, toxins, altered nutrition), thereby being proposed to act as a nutrient sensor [103-106]. Indeed, the placenta performs an active response to nutrient deprivation by suppressing nonessential energy-demanding processes. This occurs mainly by regulating the transport capacity of nutrients in response to maternal or fetal stimuli [103-106]. According to this model, in response to a maternal undernutritional status, like maternal nutrient restriction (which induces FGR), the placenta up-regulates nutrient transport (through nutrient-sensing signaling pathways) [103-106].

Interestingly enough, emerging evidence suggests that the intracellular signaling pathway mammalian target of rapamycin (mTOR) - and its up-stream regulator protein kinase B (AKT) - plays a central role as a placental nutrient-sensing mechanism [103-106]. mTOR regulates cell metabolism in response to altered nutrient levels and is influenced by a large number of upstream regulators such as amino acids [105, 107, 108], folate [105, 109], growth factors [104, 107], and free fatty acids [104, 107], which are likely to be affected by maternal nutrition [103-106]. Also, mTOR is central to the regulation of cell proliferation with input from the AKT pathway, being these pathways usually considered together as AKT-mTOR system [106].

mTOR is a serine/threonine protein kinase and comprises two major protein complexes: mTORC (mTOR complex) 1 and 2, which share the same core kinase but have different adaptor proteins, raptor and rictor, respectively [103, 110]. mTORC1 regulates cell growth, proliferation, and metabolism and mTORC2 regulates cytoskeletal organization and cellular metabolism [103]. Mechanistically, as seen in Fig. 12., mTORC1 has a direct effect upon cell proliferation due to the promotion of translation through the phosphorylation

of eukaryotic initiation factor 4E-binding proteins (4E-BP1 and 4E-BP2) and ribosomal S6 kinases (S6K1 and S6K2) [111], whereas mTORC2 activates AKT - serine/threonine protein kinase - through phosphorylation [112, 113].

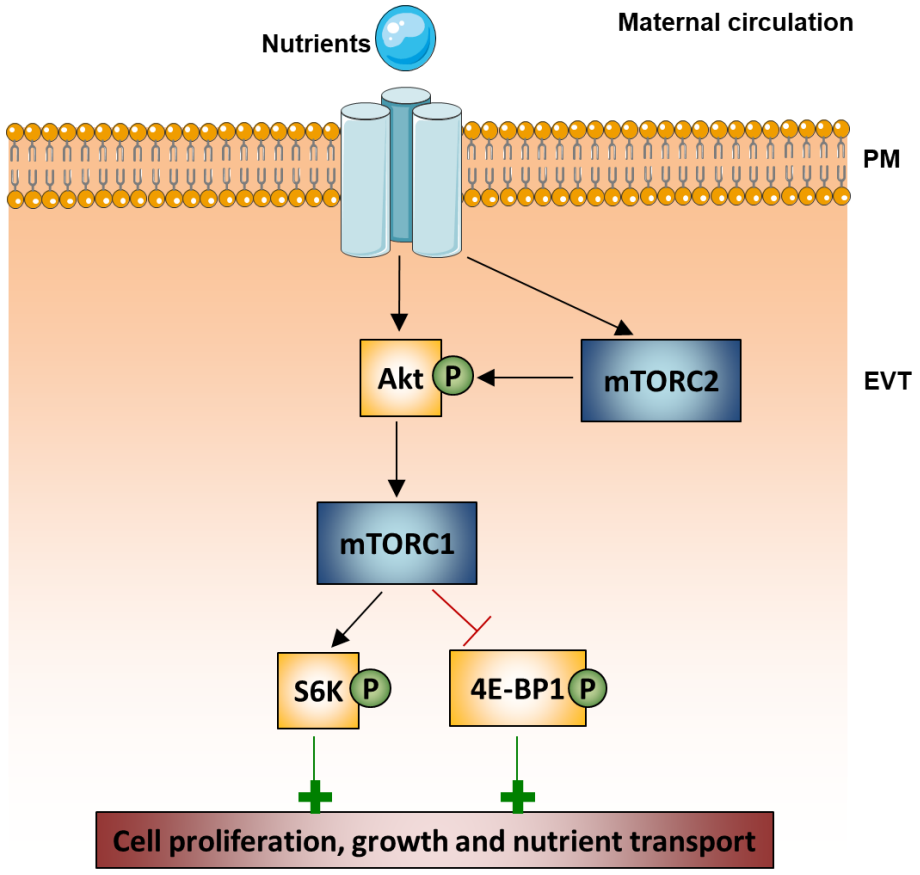


Fig. 12. Placental mTOR as a nutrient sensing signaling pathway.

mTORC2 activates AKT through phosphorylation. Akt activates mTORC1, which has a direct phosphorylation effect of 4E-BP1 and S6Ks. This seems to promote cell proliferation, growth and nutrient transport into the cell. Akt: protein kinase B; 4E-BP: 4E-binding proteins; mTORC: mTOR complex; PM: plasma membrane; S6K: ribosomal S6 kinases.

Glucose availability might regulate mTOR signaling through energy production in the form of ATP: low ATP levels (with accumulation of ADP and AMP) activate AMPK (AMP activated protein kinase) and this leads to mTOR inactivation [114], either by direct inhibitory action on raptor or by stimulation of the tuberous sclerosis complex (TSC1/2) [106], a major upstream regulator of mTORC1.

Amino acid availability also regulates mTORC1, as experiments with amino acid withdrawal, in particular L-Leu, led to inhibition of mTOR, growth restriction, and stimulation of autophagy [106]. On the other hand, the mTOR pathway in the human placenta stimulates the activity of system L [115], system A and taurine amino acid transporters [108] at the posttranslational level by influencing translocation to the apical membrane [108].

In relation to folate, mTORC1 and mTORC2 regulate cellular folate uptake in primary human trophoblast cells. Indeed, mTORC1 or mTORC2 inhibition markedly decreased the abundance of folate transporters RFC and FRα, but not PCFT [116].

In summary, the mTOR/AKT signaling pathway has been pointed as a novel regulator of placental growth and development. As reviewed by Dimasuay et. al. [103], protein restriction in rats and nutrient restriction in baboons resulted in inhibition of placental mTORC1 activity. On the other hand, placental mTOR is activated in animal models of maternal obesity and in obese women delivering large-for-gestational age (LGA) babies [103]. Also, in rats fed a low-protein diet, a reduction of mTOR activity, along with reduced insulin and AKT signaling was observed [117]. In contrast, an obesogenic diet has been shown to cause activation of mTOR in rat placentas [118], while the contrary was found after overnutrition in sheep and mice fed an obesogenic diet [119, 120]. Moreover, treatment of mouse blastocysts with rapamycin or knockout of *mTOR* leads to lethality at E5.5 associated with a failure of trophoblast outgrowth [121, 122]. Interestingly, knockout of Akt1 causes

placental and FGR in the mouse [123]. As a whole, these observations show that, although involvement of mTOR and Akt appears to exist, the nutrient sensing function of the placenta and its consequences upon the placentation process and placental growth are still not completely understood, being presently an attractive area of research.

AIMS

The main aim of this study was to investigate the effect of xenobiotics upon first-trimester placental nutrient transport and its correlation with the placentation process. We also intended to develop an experimental cellular model of insufficient human first trimester EVTs, as observed in FGR, which is presently lacking.

According to these purposes this work are divided in the following chapters:

CHAPTER I - Modulation of GLU transport and placentation-related processes in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

Given the importance of GLU as a critical nutrient for the placenta and fetal nutritional status, we intended to:

- characterize the uptake of ^{3}H-2-deoxy-D-glucose (^{3}H-DG), D-glucose analog efficiently transported by GLUT family members, by HTR-8/SVneo cells
- investigate the modulation of ^{3}H-DG uptake by exposure to several distinct xenobiotics
- investigate whether the effect of XN, EtOH and METF on uptake of ^{3}H-DG involved some specific intracellular signaling pathways
- investigate the putative correlation between modulation of uptake of ^{3}H-DG by XN and placentation-related properties (viability, proliferation, culture growth and migration) of HTR-8/SVneo cells

CHAPTER II – Modulation of LC-PUFAs transport and placentation-related processes in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

Given the importance of LC-PUFAs as essential nutrients for the placenta and fetal nutritional status, we intended to:

- characterize the uptake of ^{14}C-ARA and ^{14}C-DHA
- investigate modulation of ^{14}C-ARA and ^{14}C-DHA uptake by XN
- investigate whether the effect of XN on uptake of ^{14}C-ARA involved some specific intracellular signaling pathways
- study the putative correlation between modulation of uptake of ^{14}C-ARA by XN and placentation-related properties (viability, proliferation, culture growth, migration and apoptosis) of HTR-8/SVneo cells

CHAPTER III – Modulation of FA transport in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

Given the importance of FA as a critical nutrient for the placenta and fetal nutritional status, we intended to:

- characterize the uptake of ^{3}H-FA
- investigate the modulation of uptake of ^{3}H-FA by XN, EtOH and METF
- investigate whether the effect of XN, EtOH and METF on uptake of ^{3}H-FA involved some specific intracellular signaling pathways

CHAPTER IV – Modulation of L-MET transport in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

Given the importance of L-MET as a critical nutrient for the placenta and fetal nutritional status, we intended to:

- characterize the uptake of ^{14}C-L-MET
- investigate the modulation of uptake of ^{14}C-L-MET by XN

CHAPTER V – Modulation of placentation-related processes in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

Given that the placenta is exposed to a plethora of xenobiotics that might alter nutrient transport as well as the process of placentation, we intended to:

- investigate modulation of placentation-related processes such as cell viability, proliferation, culture growth, migration and apoptosis index by a plethora of xenobiotics
- investigate the involvement of some intracellular signaling pathways on the cytotoxic and antiproliferative effect of EtOH and METF

CHAPTER I

Modulation of GLU transport and placentation-related processes in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

The information contained in this chapter is included in the following original publications:

Manuscript A. Correia-Branco A, Azevedo CF, Araujo JR, Guimaraes JT, Faria A, Keating E and Martel F. **Xanthohumol impairs glucose uptake by a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) and impacts the process of placentation.** Molecular Human Reproduction, 2015, 21(10):803-815

Molecular Human Reproduction (Mol Hum Reprod)
DOI: 10.1093/molehr/gav043
IF: 3.585

Manuscript B. Correia-Branco A, Keating E and Martel F. **Involvement of mTOR, JNK and PI3K in the negative effect of ethanol and metformin on the human first-trimester extravillous trophoblast HTR-8/SVneo cell line**. European Journal of Pharmacology, 2018 (accepted).

European Journal of Pharmacology (Eur J Pharmacol)
DOI: 10.1093/molehr/gav043
IF: 2.896

Manuscript A. Correia-Branco A, Azevedo CF, Araujo JR, Guimaraes JT, Faria A, Keating E and Martel F. **Xanthohumol impairs glucose uptake by a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) and impacts the process of placentation.** Molecular Human Reproduction, 2015, 21(10):803-815.

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molecular human reproduction

ORIGINAL RESEARCH

Xanthohumol impairs glucose uptake by a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) and impacts the process of placentation

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ABSTRACT: In this study, we aimed to investigate modulation of glucose uptake by the HTR-8/SVneo human first-trimester extravillous trophoblast cell line by a series of compounds and to study its consequences upon cell proliferation, viability and migration. We observed that uptake of ^{3}H-deoxy-D-glucose (^{3}H-DG; 10 nM) was time-dependent, saturable, inhibited by cytochalasin B (50 and 100 μM), phloretin (0.5 mM) and phloridzin (1 mM), insulin-insensitive and sodium-independent. In the short term (30 min), neither 5-HT (100–1000 μM), melatonin (10 nM) nor the drugs of abuse ethanol (100 mM), nicotine (100 μM), cocaine (25 μM), amphetamine (10–25 μM) and 3,4-methylenedioxy-*N*-methamphetamine (10 μM) affected ^{3}H-DG uptake, while dexamethasone (100–1000 μM), fluoxetine (100–300 μM), quercetin, epigallocatechin-3-gallate (30–1000 μM), xanthohumol (XH) and resveratrol (1–500 μM) decreased it. XH was the most potent inhibitor [IC_{50} = 3.55 (1.37–9.20) μM] of ^{3}H-DG uptake, behaving as a non-competitive inhibitor of ^{3}H-DG uptake, both after short- and long-term (24 h) treatment. The effect of XH (5 μM; 24 h) upon ^{3}H-DG uptake involved mammalian target of rapamycin, tyrosine kinases and c-Jun N-terminal kinases intracellular pathways. Moreover, XH appeared to decrease cellular uptake of lactate due to inhibition of the monocarboxylate transporter 1. Additionally, XH (24 h; 5 μM) decreased cell viability, proliferation, culture growth and migration. The effects of XH upon cell viability and culture growth, but not the antimigratory effect, were mimicked by low extracellular glucose conditions and reversed by high extracellular glucose conditions. We thus suggest that XH, by inhibiting glucose cellular uptake and impairing HTR-8/SVneo cell viability and proliferation, may have a deleterious impact in the process of placentation.

Key words: glucose uptake / proliferation / viability / trophoblast cells / xanthohumol

Introduction

The placenta provides the interface between the fetal and maternal environments, being involved in the exchange of gases, nutrients and waste products between the mother and the growing fetus (Cross, 1998; Fowden *et al.*, 2008), serving as an endocrine organ by producing several pregnancy-associated hormones and growth factors and ensuring the protection of the fetus from maternal immune attack (Regnault *et al.*, 2002). Early after fertilization, the process of blastocyst implantation begins along with differentiation of trophoblastic stem cells into two cell lineages: (a) villous cytotrophoblasts that fuse to form the multinucleated syncytiotrophoblasts, which ensure placental endocrine, protective and transport functions, and (b) extravillous trophoblasts (EVTs), which are the main participants in the process of placentation (Vicovac *et al.*, 1995; Ji *et al.*, 2013). Placentation is a continuous and highly regulated process that begins right after fertilization and ends only after delivery (James *et al.*, 2012). An adequate placentation and a consequent adequate nutrient supply to the fetus are crucial factors

for fetal development and growth and for pregnancy outcome. Such critical importance is evident as fetal growth restriction (FGR) is commonly characterized by impaired uterine blood flow and placental development causing reduced fetal nutrient uptake as well as fetal hypoxia (James et al., 2012). EVTs are fully specialized trophoblasts exhibiting an invasive and proliferative phenotype. They perform the anchorage of the chorionic villi into the uterine wall and actively regulate the process of uterine spiral arteries remodeling, a process that ends up with the establishment of the utero-placental blood flow (Ji et al., 2013).

Glucose is a major substrate for fetal and placental energy metabolism. However, gluconeogenesis in the feto-placental unit is minimal (Magnusson et al., 2004). Therefore, the supply of glucose from the maternal circulation is mandatory for the process of placentation and for the normal fetal development and growth (Baumann et al., 2002; Carter, 2012). Despite such importance, glucose homeostasis in first-trimester trophoblasts and the implications of this mechanism in the process of placentation are still largely unexplored. Glucose transport at the cell membrane level involves two structurally and functionally distinct families of transporters, namely sodium-dependent glucose co-transporters (SGLTs, gene symbol SLC5A) (Wright, 2001) and facilitative Na^+-independent sugar transporters [glucose transporter (GLUT) family, gene symbol SLC2A], a group comprising at least 12 isoforms (GLUT1–GLUT12) (Illsley, 2000). Among the 12 isoforms, GLUT1 is a ubiquitous isoform, expressed in almost all tissues and it is regarded as the constitutive form of the glucose transporter in the placenta from implantation to term (Illsley, 2000). Pregnant women are exposed to several bioactive compounds including: (a) polyphenols through ingestion of vegetables, fruits and beverages (Manach et al., 2004), (b) therapeutic compounds, since many pregnant women take medication for the treatment of various conditions such as epilepsy, hypertension, depression, human immunodeficiency virus and gestational diabetes and (c) drugs of abuse and alcohol, which are known to exert deleterious effects on the fetus (Ganapathy, 2011).

Nothing was known concerning the characteristics of glucose uptake by first-trimester EVT cells and the implications of this mechanism in the placentation process; therefore, we herein intended to study glucose uptake in a human first-trimester EVT cell line (HTR-8/SVneo cells), its modulation by several compounds, such as dietary compounds, therapeutic agents and drugs of abuse, and whether the modulation of glucose transport by these compounds interferes with the placentation process.

Materials and Methods

Materials

The materials listed below were used in the study.

^{3}H-2-deoxy-D-glucose (2-[1,2-^{3}H(N)]-deoxy-D-glucose; ^{3}H-DG; specific activity 50 Ci/mmol) (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA); ^{3}H-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH, Freiburg, Germany); ^{14}C-*n*-butyric acid ([1-^{14}C]-*n*-butyric acid, sodium salt; ^{14}C-BT; specific activity 30–60 mCi/mmol) (Biotrend Chemikalien GmbH, Köln, Germany); amphetamine, antibiotic/antimicotic solution (100 U ml^{-1} penicilin, 100 µg ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B), ascorbic acid, caffeine (CAF), cocaine, cytochalasin B (CYT B; from *Drechslera dematioidea*), DG, dexamethasone (DEXA), epigallocatechin gallate (EGCG), fluoxetine hydrochloride (FLUOX), genistein, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), human recombinant insulin, LY294002 hydrochloride, MDMA (3,4-methylenedioxy-*N*-methamphetamine), melatonin (MELAT; *N*-acetyl-5-methoxytryptamine), MES (*N*-2-hydromorfolin-ethanesulfonic acid), NADH (reduced nicotinamide adenine dinucleotide), (−)-nicotine hydrogen tartrate salt, pargyline hydrochloride, PD98059, phloridzin (PHZ), quercetin (QUE), resveratrol (RESV), RPMI 1640 medium, serotonin creatinine sulfate monohydrate (5-HT), SP600125, sulforhodamine B, theophylline (TEO), trichloroacetic acid (TCA), Tris, triton X-100 and trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma, St. Louis, MO, USA). Fetal bovine serum (Gibco, Life Technologies Corporation, CA, USA), DMSO (dimethylsulfoxide), random hexamer primers, RNAse H, superscript reverse transcriptase II (Invitrogen Corporation, CA, USA), D-glucose, Triton X-100 (Merck, Dramstad, Germany), phloretin (PHT; Santa Cruz Biotechnology, CA, USA); RNA-STAT 60^{TM} isolation reagent (Amsbio, AMS Biotechnology Limited, UK); qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA); KAPA SYBR® FAST quantitative polymerase chain reaction (qPCR) Master Mix (KAPA SYBR® FAST qPCR Kit Master Mix Universal, Kapa Biosystems, Wilmington, MA, USA). XH was kindly donated by Eng. José M. Machado Cruz (Instituto de Bebidas e Saúde (iBeSa), S. Mamede Infesta, Portugal).

The drugs to be tested were dissolved in water (ascorbic acid, cocaine, DG, FLUOX, MELAT, NADH, (−)-nicotine, pargyline, 5-HT), 1% (v/v) acetic acid (sulforhodamine B), 100% (v/v) ethanol (DEXA, PHZ, XH), 100% (v/v) methanol (amphetamine, MDMA, PHT), DMSO (CAF, CYT B, EGCG, genistein, LY 294002, PD 98059, QUE, rapamycin, RESV, SB 203580, SP 600125 and TEO) or 0.01 M HCl (insulin). The final concentration of these solvents was 1% (v/v) in pre-incubation and incubation buffer and in the culture media. Controls for these compounds were run in the presence of the respective solvents. Stock solutions (amphetamine 7.4 mM, cocaine 100 mM, CYT B 10 mM, EGCG 100 mM, genistein 100 mM, insulin 0.1 mM, LY 294002 10 mM, MDMA 5.2 mM, pargyline 10 mM, PD 98059 10 mM, PHT 100 mM, PHZ 100 mM, QUE 100 mM, rapamycin 1 mM, RESV 100 mM, SB 203580 10 mM, 5-HT 100 mM, SP 600125 10 mM and XH 100 mM) were stored at −20°C, unless otherwise stated.

Human first-trimester EVT cell culture (HTR-8/SVneo cells)

HTR-8/SVneo cells were generously provided by Dr Charles H. Graham (Department of Anatomy & Cell Biology, Queen's University at Kingston, Canada) (Graham et al., 1993) and were used between passage number 72 and 86. Cells were maintained in a humidified atmosphere of 5% CO_2—95% air and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution. The culture medium was changed every 2–3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.05% trypsin-EDTA, 5 min, 37°C), split 1:6 ratio and sub-cultured in plastic culture dishes (21 cm^2; diameter 60 mm, TPP®, Trasadingen, Switzerland). For transport experiments, for glucose metabolism assessment and for quantification of cell viability and migration, HTR-8/SVneo cells were seeded on 24-well plastic cell culture dishes (2 cm^2; Ø 16 mm; TPP®), and were used after 7–11 days in culture (90–100% confluence). For proliferation and culture growth assays, cells were seeded on 24-well plastic cell culture dishes (2 cm^2; Ø 16 mm; TPP®), and were used after 5 days in culture (70–80% confluence). For RNA and protein extraction, cells were seeded in plastic culture dishes (21 cm^2; Ø 60 mm; TPP®) and were used after 7–11 days in culture (90–100% confluence). The cell medium was free of fetal calf serum for 24 h before the experiments.

Transport studies

Transport experiments were performed in glucose-free-HEPES-buffered saline (GF-HBS) buffer (composition (all mM): 20 HEPES–NaOH, 5 KCl, 140 NaCl, 2.5 $MgCl_2$, 1 $CaCl_2$ and pH 7.4). Initially the culture medium

was aspirated and the cells were washed with buffer at 37°C; then the cell monolayers were preincubated for 20 min with buffer at 37°C. Uptake was initiated by the addition of 0.2 ml buffer at 37°C containing 10 or 20 nM ^{3}H-DG (except in experiments for determination of kinetics of ^{3}H-DG uptake). Incubation was stopped after 10 min (except in the time-course experiments) by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml ice-cold buffer). In experiments for determination of kinetics of ^{3}H-DG uptake, cells were incubated for 10 min with increasing concentrations (0.01–10 000 μM) of ^{3}H-DG. In ^{14}C-BT (10 μM) uptake experiments, an incubation time of 3 min was used. At the end of the incubation period, the cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM Tris–HCl, pH 7.4), and placed at 4°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Treatment of the cells

The concentrations of compounds to be tested were chosen based on previous work of our group (Araújo *et al.*, 2008). In order to test the short-term effect of compounds, cells were exposed to the compounds in GF-HBS buffer during the pre-incubation and incubation periods (corresponding to 30 and 23 min exposure times in ^{3}H-DG and ^{14}C-BT experiments, respectively). In order to test the long-term effect of compounds, cells were exposed to the compounds for 24 h in serum-free culture medium, and during the pre-incubation and incubations periods in GF-HBS buffer (corresponding to 30 and 23 min exposure times in ^{3}H-DG and ^{14}C-BT experiments, respectively). In some experiments, long-term treatment of the cells with XH (5 μM; 24 h) was performed in GF-HBS buffer with different concentrations of D-glucose (1 , 11 and 20 mM).

Protein determination

The protein content of cell monolayers was determined as described by Bradford (1976), using human serum albumin as standard.

Determination of cell viability

The short- and long-term effect of compounds upon the viability of HTR-8/SVneo cells was determined spectrophotometrically by measuring the cellular leakage of the cytosolic enzyme lactate dehydrogenase (LDH) into the extracellular medium. This was done by quantification of the decrease in absorbance of NADH during the reduction of pyruvate to lactate, as described by Bergmeyer and Bernt (1974).

Determination of cell proliferation rates

Cell proliferation rates were determined with the ^{3}H-thymidine incorporation assay. Briefly, HTR-8/SVneo cells treated for 24 h with XH (or the respective solvent) were incubated with ^{3}H-thymidine 0.025 μCi/ml during the last 5 h of the 24-h incubation period. After removal of excess ^{3}H-thymidine by a 10% TCA (300 μl) wash for 1 h at 4°C, drying for 30 min and addition of 280 μl of NaOH 1 M, the incorporated ^{3}H-thymidine was measured by liquid scintillometry.

Determination of culture growth

Culture growth was determined by the sulforhodamine B (SRB) assay. Briefly, HTR-8/SVneo cells were treated for 30 min or 24 h with XH (or the respective vehicle). At the end of treatment, 62.5 μl of ice-cold 50% (w/v) TCA were added to the culture medium (500 μl) in each well to fix cells (1 h at 4°C in the dark). The plates were then washed five times with tap water to remove TCA. Plates were air-dried and then stained for 15 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid. SRB was removed, and cultures were rinsed four times with 1% (v/v) acetic acid to remove residual dye. Plates were again air-dried, and the bound dye was then solubilized with 375 μl of 10 mM Tris–NaOH solution (pH 10.5). The absorbance of each well was determined at 540 nm; samples were diluted to obtain absorbance values <0.7.

Determination of migration rates

Cell migration rate was determined by the *in vitro* wound healing assay. Briefly, HTR-8/SVneo cell monolayers were scratched with a 10 μl pipette tip and were afterwards treated for 24 h with XH (or the respective vehicle). Images were obtained at 0, 6, 12 and 24 h of treatment and quantification was performed by using the ImageJ software (Reinhart-King, 2008; Negrão *et al.*, 2013).

Glucose metabolism assessment

Glucose metabolism was assessed by quantification of lactate in the extracellular medium of HTR-8/SVneo cells treated with XH (or vehicle) for 30 min or 24 h. At the end of treatment, extracellular medium was collected and centrifuged at 8000 × g for 10 min. Lactate concentration was then measured by the lactate oxidase/peroxidase colorimetric assay, as indicated by the manufacturer (Olympus Life and Material Science Europa GmbH, Hamburg, Germany).

RNA extraction and real-time qPCR

Total RNA was extracted from HTR-8/SVneo cells treated for 24 h with XH (or vehicle), using RNA-STAT 60™ isolation reagent, according to manufacturer's instructions (Amsbio, AMS Biotechnology (Europe) Limited, UK). Before cDNA synthesis, total RNA was treated with DNAse I (Ambion, Inc., TX, USA) and 1 μg of the resulting DNA-free RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) in 20 μl of final reaction volume, according to manufacturer's instructions. For real-time qPCR (qRT–PCR), 2 μl of the 20 μl reverse transcription reaction mixture was used. For the calibration curve, HTR-8/SVneo standard cDNA was diluted in six different concentrations. qRT–PCR was carried out using a LightCycler® 96 (Roche, Nutley, NJ, USA). Ten microliter reactions were setup in 96-well plates using 10 μM of each primer and 5 μl of KAPA SYBR® FAST qPCR Master Mix (KAPA SYBR® FAST qPCR Kit Master Mix Universal, Kapa Biosystems, Wilmington, MA, USA), according to manufacturer's instructions. Cycling conditions for human GLUT1 amplification were as follows: denaturation (95°C for 5 min), amplification and quantification [95°C for 10 s, annealing temperature (AT) for 10 s, and 65°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment] repeated 55 times, followed by a melting curve program [(AT + 10)°C for 15 s and 75°C with a heating rate of 0.1°C/s and continuous fluorescence measurement] and a cooling step to 37°C. The primer pair used for human GLUT1 amplification was 5′-GAT GAT GCG GGA GAA GAA GGT-3′ (forward) and 5′-ACA GCG TTGA TGC CAG ACA G-3′ (reverse). The amount of GLUT1 mRNA was normalized to the amount of mRNA of the housekeeping gene, human β-actin. Cycling conditions for human β-actin amplification were as follows: denaturation (95°C for 5 min), amplification and quantification (95°C for 10 s, AT for 10 s, and 65°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment) repeated 45 times, followed by a melting curve program [(AT + 10)°C for 15 s and 75°C with a heating rate of 0.1°C/s and continuous fluorescence measurement] and a cooling step to 37°C; the primer pair used for β-actin was: 5′-AGA GCC TCG CCT TTG CCG AT-3′ (forward) and 5′-CCA TCA CGC CCT GGT GCC T-3′ (reverse). Data were analyzed using the LightCycler® 96 SW 1.1 analysis software (Roche, Mannheim, Germany), and results analyzed by the Ct method (Schmittgen and Livak, 2008). β-Actin mRNA expression levels were not affected by treatment of the cells with XH (data not shown).

Western blotting assay

Proteins were extracted from HTR-8/SVneo cells previously treated for 24 h with vehicle or XH, using RIPA isolation reagent, according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Protein concentration was quantified using the Pierce BCA Protein Assay Kit. Equal amounts of protein (20 μg of protein) were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis with a 5% stacking gel. After electrophoresis, proteins were blotted into a Hybond nitrocellulose membrane (Hybond™ -C extra, Amersham Biosciences, Hammersmith, UK), using a mini-transblot electrophoretic transfer cell (Amersham Biosciences, Pittsburg, PA, USA). Immunodetection for GLUT1 and β-actin (Santa Cruz Biotechnology, Inc., CA, USA), was accomplished with enhanced chemiluminescence (ECL kit, Amersham Biosciences, Pittsburg, PA, USA). The relative intensity of each protein was measured using a computerized software program (Biorad, CA, USA) and normalized with β-actin bands to compare the expression of proteins in the different treatment groups.

Calculations and statistics

For the analysis of the time-course of ^{3}H-DG uptake, the parameters of the equation ($A(t) = k_{in}/k_{out}(1 - e^{-k_{out}t})$) were fitted to the experimental data by a non-linear regression analysis, using a computer-assisted method (Muzyka *et al.*, 2009). $A(t)$ represents the accumulation of ^{3}H-DG at time t, k_{in} and k_{out} the rate constants for inward and outward transport, respectively, and t the incubation time. A_{max} is defined as the accumulation at steady state ($t \rightarrow \infty$). For the analysis of the saturation curve, the parameters of the Michaelis–Menten equation were fitted to the experimental data by using a non-linear regression analysis, using a computer-assisted method (Muzyka *et al.*, 2009). For calculation of IC_{50} values, corresponding to the concentration of drug causing 50% of its maximal effect, the parameters of the Hill equation were fitted to the experimental data by using a non-linear regression analysis, using a computer-assisted method (Muzyka *et al.*, 2009).

Arithmetic means are given with SEM and geometric means with 95% confidence intervals. The value n represents the number of replicates of at least two different experiments. A statistical significance of the difference between two groups was evaluated by the Student's t-test and statistical significance of the difference between three or more groups was evaluated by one-way analysis of variance, followed by the Bonferroni test (GraphPad Prism, Graphpad Software, Inc., La Jolla, CA, USA). Differences were considered to be significant when $P < 0.05$.

Results

^{3}H-DG uptake is time-dependent and saturable

In a first series of experiments, we determined the time-course of ^{3}H-DG accumulation by HTR-8/SVneo cells. As shown in Fig. 1, uptake of ^{3}H-DG (20 nM) by HTR-8/SVneo cells was time-dependent, being linear with time for up to 10 min of incubation (Fig. 1 inset), after which uptake reached a plateau. Thus, in subsequent experiments, initial rates of ^{3}H-DG uptake were measured by incubating cells for 10-min with ^{3}H-DG. The kinetics of ^{3}H-DG uptake were also determined. ^{3}H-DG (1–10 000 μM) uptake was saturable, with the following kinetic parameters: V_{max} of 63 ± 4.8 nmol mg prot^{-1} 10 min^{-1} and K_m of 2.9 ± 0.5 mM.

^{3}H-DG uptake is mediated by members of the facilitative glucose transporters family

In order to investigate the specificity of the carrier process involved in ^{3}H-DG uptake by HTR-8/SVneo cells, the effects of CYT B and PHT, inhibitors of GLUT transporters, and of PHZ, an inhibitor of the SGLT1 which also appears to inhibit GLUT transporters (Gonçalves*et al.*, 2008; Moreira *et al.*, 2013), upon ^{3}H-DG uptake were determined. As shown in Fig. 2A, ^{3}H-DG uptake by HTR-8/SVneo cells was inhibited by CYT B, PHT and PHZ. Because ^{3}H-DG uptake was not reduced when extracellular sodium was replaced by Li^+ (Fig. 2B), the putative involvement of SGLT1 in ^{3}H-DG uptake was excluded, and it can be concluded that ^{3}H-DG uptake by HTR-8/SVneo cells involves a GLUT transporter. Finally, the lack of effect of insulin (Fig. 2C), which his known to stimulate GLUT4- and GLUT12-mediated glucose transport, excludes the involvement of these transporters. We thus conclude that ^{3}H-DG uptake by these cells involve a member of the GLUT family of transporters distinct from GLUT4 and GLUT12, which most probably corresponds to GLUT1.

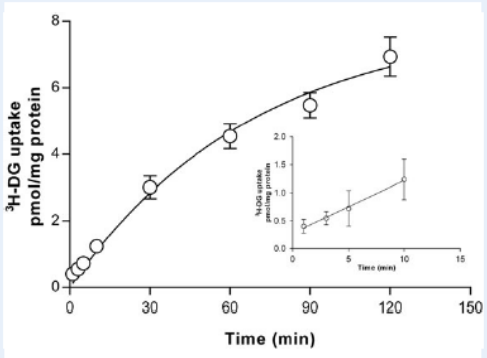

Figure 1 Time-course of ^{3}H-DG uptake by HTR-8/SVneo cells. HTR-8/SVneo cells (a human first-trimester extravillous trophoblast cell line) were incubated at 37°C, pH7.4, with 20 nM ^{3}H-deoxy-D-glucose (^{3}H-DG) for various periods of time ($n = 12$). Inset highlights linearity of uptake for the first 10 min of incubation. Shown are arithmetic mean ± SEM.

Several distinct compounds interfere with ^{3}H-DG uptake

Therapeutic drugs, drugs of abuse and hormones

The short-term (30 min) effect of some drugs of abuse (nicotine (100 μM), cocaine (25 μM), MDMA (10 μM), amphetamine (25 μM) and ethanol (100 mM)) on ^{3}H-DG uptake was investigated. None of these drugs had any significant effect upon ^{3}H-DG uptake (data not shown). Interestingly, short-term FLUOX (100–300 μM) reduced ^{3}H-DG uptake. This effect is not related to an increase in 5-HT concentration because serotonin (5-hydroxytryptamine; 5-HT) itself was devoid of effect (Fig. 3A). Moreover, DEXA reduced ^{3}H-DG uptake in a concentration-independent manner, while MELAT did not affect it (Fig. 3B).

Dietary compounds

The short-term effect of some dietary polyphenols (QUE, EGCG, XH and RESV) and methylxanthines (CAF and TEO) on ^{3}H-DG uptake by HTR-8/SVneo cells was also assessed. Interestingly, all these compounds significantly reduced ^{3}H-DG uptake, with the polyphenols

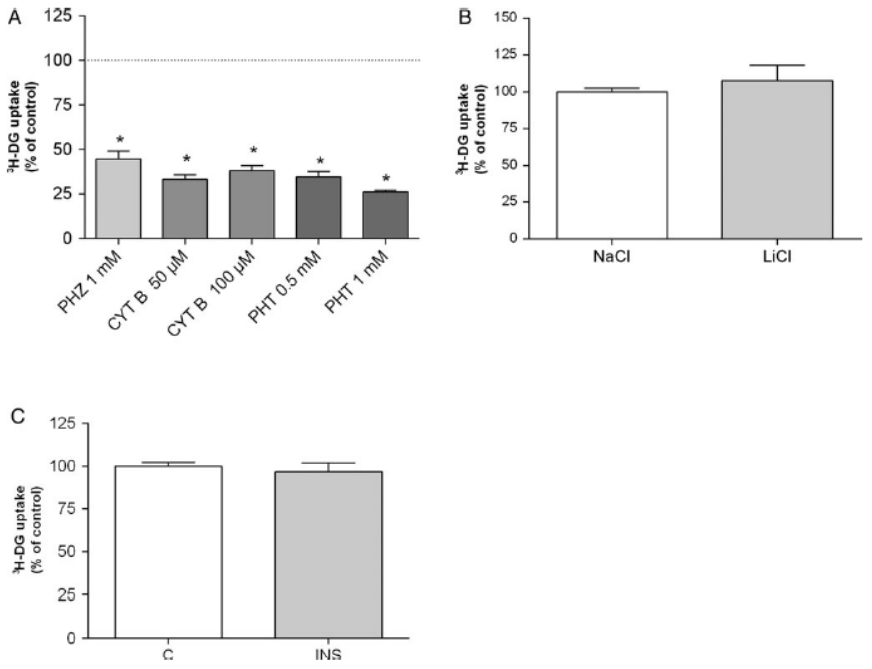

Figure 2 Characterization of ^{3}H-DG uptake by HTR-8/SVneo cells. Cells were preincubated for 20 min and then incubated with 10 nM ^{3}H-DG for 10 min: (**A**) in the presence of 1 mM phloridzin (PHZ; $n = 10$), 50 or 100 µM cytochalasin B (CYT B; $n = 5–10$), 0.5 or 1 mM phloretin (PHT; $n = 5–10$) or the respective solvents (control, indicated by the dashed line; $n = 10$); (**B**) in buffer containing sodium chloride (NaCl; $n = 9$) or an isotonic concentration of lithium chloride (LiCl; $n = 9$); or (**C**) in the presence of insulin 100 nM (INS; $n = 9$) or the respective solvent (control, C; $n = 9$). Shown are arithmetic means ± SEM. *$P < 0.05$ significantly different from control (Student's t test).

presenting a very marked and concentration-dependent inhibitory effect (Fig. 4). The inhibitory effect of the polyphenols was not related to a decrease in cell viability, as none of them, when tested in the highest concentration (QUE and EGCG: 1000 µM; XH and RESV: 500 µM), showed a cytotoxic effect in the LDH leakage assay (control: 24.1 ± 4.9% total; QUE: 21.9 ± 5.4% of total; EGCG: 26.2 ± 4.2% of total; XH: 31.6 ± 7.6% of total and RESV: 20.6 ± 5.0% of total; $n = 7–8$). In the presence of CYT B (50 µM), an inhibitor of GLUT-mediated glucose transport, none of the polyphenols was able to further decrease the uptake of ^{3}H-DG (Fig. 5), suggesting that these dietary compounds inhibit GLUT-mediated ^{3}H-DG uptake.

XH is a potent non-competitive inhibitor of ^{3}H-DG uptake

We next determined the potency of the polyphenols in relation to inhibition of ^{3}H-DG uptake by HTR-8/SVneo cells. Analysis of inhibition curves after short-term exposure to the polyphenols allowed the calculation of IC_{50} values for RESV, XH and EGCG, which are displayed in Fig. 6A and Table I. As shown in Table I, XH was the most potent inhibitor of ^{3}H-DG uptake by HTR-8/SVneo cells. The effect of short-term exposure to XH upon the kinetic parameters of ^{3}H-DG uptake by HTR-8/SVneo cells was also evaluated. XH was found to be a non-competitive inhibitor ^{3}H-DG uptake by HTR-8/SVneo cells (Table II). A longer-term (24 h) exposure of the cells to XH (0.03–5 µM) was also tested. Although with a lower potency, this polyphenol was also able to reduce ^{3}H-DG uptake by these cells (Fig. 6B) and it behaved (5 µM; 24 h) as a non-competitive inhibitor of ^{3}H-DG uptake as well (results not shown)

Inhibition of ^{3}H-DG uptake by XH involves mTOR, TK and c-JNK signaling pathways

Interestingly enough, the effect of XH (5 µM; 24 h) upon ^{3}H-DG uptake was reversed by genistein (10 µM), SP 600125 (5 µM) and rapamycin (100 nM), indicating the involvement of tyrosine kinases (TKs), c-Jun N-terminal kinases (JNK) and mammalian target of rapamycin (mTOR) intracellular pathways, respectively (Fig. 7). The involvement of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) was excluded due to the lack of efficiency of LY 294002 in reversing the effect of XH.

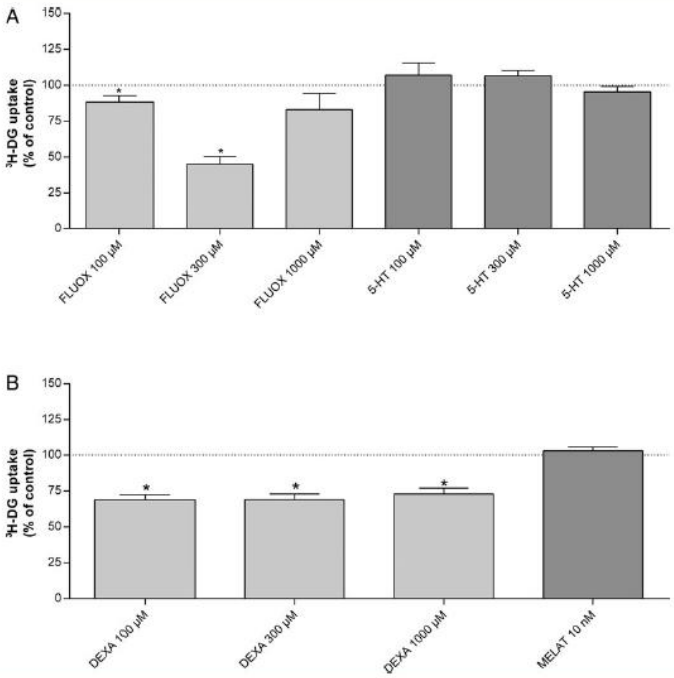

Figure 3 ^{3}H-DG uptake by HTR-8/SVneo cells is inhibited by short-term exposure to fluoxetine and dexamethasone. Cells were preincubated for 20 min and then incubated with 10 nM ^{3}H-DG for 10 min: (**A**) in the presence of 100–1000 µM fluoxetine (FLUOX; $n = 3–16$), 100–1000 µM serotonin (5-HT; $n = 3$) or the respective solvents (control, indicated by the dashed line; $n = 12–13$); or (**B**) in the presence of 100–1000 µM dexamethasone (DEXA; $n = 9–12$), 10 nM melatonin (MELAT; $n = 6$) or the respective solvents (control, indicated by the dashed line; $n = 12–15$). Shown are arithmetic means ± SEM. *$P < 0.05$ significantly different from control (Student's t test).

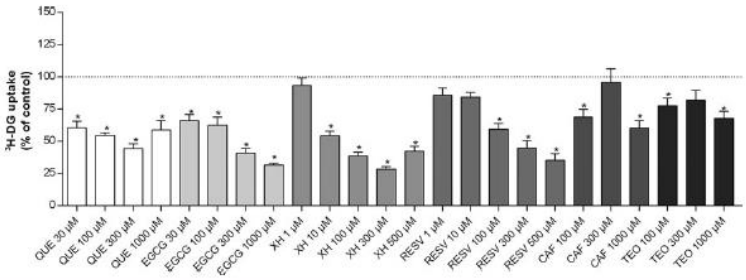

Figure 4 ^{3}H-DG uptake by HTR-8/SVneo cells is inhibited by short-term exposure to dietary polyphenols and methylxanthines. Cells were preincubated for 20 min and then incubated with 10 nM ^{3}H-DG for 10 min in the presence of 30–1000 µM quercetin (QUE; $n = 6–15$), 30–1000 µM epigallocatechin-3-gallate (EGCG; $n = 6–15$), 1–500 µM xanthohumol (XH; $n = 8–10$), 1–500 µM resveratrol (RESV; $n = 8–11$), 100–1000 µM caffeine (CAF; $n = 11–12$) or 100–1000 µM theophylline (TEO; $n = 11–12$) or the respective solvents (control, indicated by the dashed line; $n = 11–30$). Shown are arithmetic means ± SEM. *$P < 0.05$ significantly different from control (Student's t test).

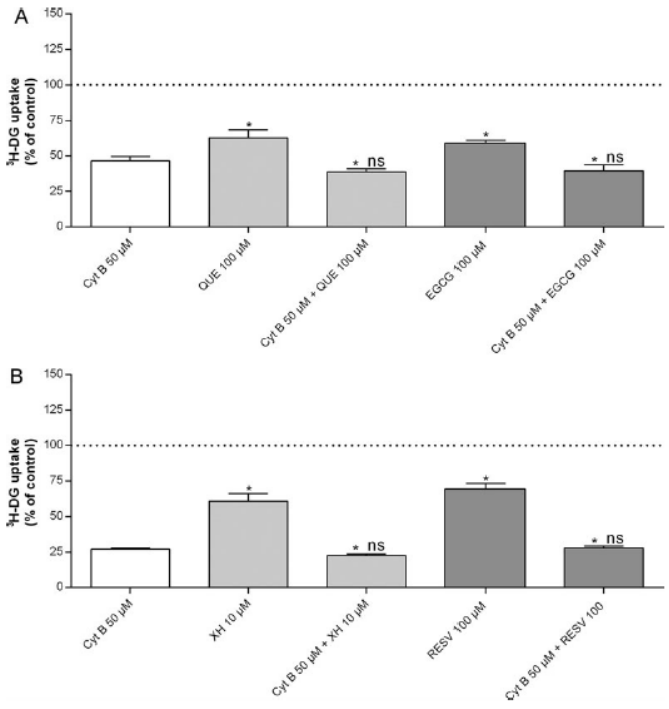

Figure 5 Dietary polyphenols inhibit GLUT-mediated ^{3}H-DG uptake by HTR-8/SVneo cells. Cells were preincubated for 20 min and then incubated with 10 nM ^{3}H-DG for 10 min in the presence of: (**A**) 100 µM QUE ($n = 8$), 100 µM EGCG ($n = 8$); or (**B**) 10 µM XH ($n = 8$) or 100 µM RESV ($n = 8$), either alone or in the presence of 50 µM CYT B ($n = 8$) or the respective solvent (control, indicated by the dashed line; $n = 8$). Shown are arithmetic means ± SEM. *$P < 0.05$ significantly different from control. ns = not significantly significant from 50 µM Cyt B (one-way analysis of variance followed by Bonferroni test).

Changes in GLUT1 gene expression and protein levels do not seem to underlie the inhibitory effect of XH upon ^{3}H-DG uptake

XH (5 µM; 24 h) did not alter GLUT1 gene expression (mRNA) levels (Supplementary data, Fig. S1A), and although we found a tendency for a decrease in GLUT1 protein levels, it was not statistically significant (Supplementary data, Fig. S1B).

XH interferes with monocarboxylate transporter 1-mediated lactate cellular uptake

Knowing that XH reduces ^{3}H-DG cellular uptake by HTR-8/SVneo cells, interference of cellular glucose metabolism by this compound was next investigated, by quantifying lactate levels. Surprisingly, a short-term (30 min) exposure to XH (100 µM) led to an increase in the extracellular concentration of lactate (from 100 ± 4% to 133 ± 6% of control; $n = 8$), which was much more pronounced after a longer exposure of the cells to this compound (24 h; XH 5 µM) (from 100 ± 1% to 285 ± 21% of control; $n = 8$). We hypothesized that XH is interfering with monocarboxylate transporter 1 (MCT1)-mediated cellular re-uptake of released lactate. Confirmation of this hypothesis was obtained with the observation that short-term XH (30 min; 100 µM) significantly reduced the cellular uptake of a well-known MCT1 substrate, ^{14}C-BT (10 µM) (to 54 ± 5% of control, $n = 12$) (data not shown).

XH exerts antiproliferative, cytotoxic and antimigratory effects

A 24 h-exposure of HTR-8/SVneo cells to XH (5 µM) induced a very marked reduction in cell proliferation rate (Fig. 8A), cell viability (Fig. 8B), cell culture growth (Fig. 8C) and cell migration capacity (Fig. 8D and E).

The antiproliferative and cytotoxic effect of XH is dependent on inhibition of glucose uptake

Interestingly, low extracellular glucose conditions were able to mimic the effect of XH (5 µM) upon cell viability and culture growth (Fig. 9A and B), suggesting that the antiproliferative and cytotoxic effect of XH is dependent on inhibition of glucose entry and thus related to glucose

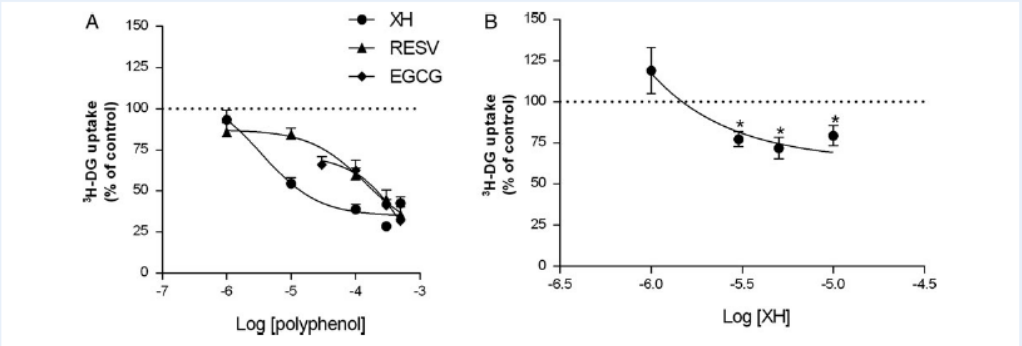

Figure 6 ^{3}H-DG uptake by HTR-8/SVneo cells is inhibited by short- and long-term exposure to some dietary polyphenols. (**A**) Cells were preincubated for 20 min and then incubated with 10 nM ^{3}H-DG for 10 min in the presence of 30–1000 μM EGCG ($n = 6–15$), 1–500 μM XH ($n = 8–10$) or 1–500 μM RESV ($n = 8–11$); (**B**) cells were preincubated for 24 h in serum-free culture medium and then incubated with 10 nM ^{3}H-DG for 10 min in the presence of 0.03–5 μM XH ($n = 8–10$) or the respective solvent (control, indicated by the dashed line; $n = 11–30$). Shown are arithmetic means ± SEM. *$P < 0.05$ significantly different from control (Student's t test).

Table I Inhibition of ^{3}H-deoxy-D-glucose (^{3}H-DG) uptake in a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) by short-term exposure to epigallocatechin-3-gallate (EGCG), xanthohumol (XH) and resveratrol (RESV).

	IC_{50} (μM)	95% CI	n
XH	3.55	1.37–9.20	8–10
RESV	141.5	40.7–492.7	8–11
EGCG	932	5.69–1528	6–15

CI: 95% confidence intervals.
IC_{50}: the concentration of drug causing 50% of its maximal effect.

Table II Kinetic parameters of ^{3}H-DG uptake by HTR-8/SVneo cells in the absence (control) and presence of a short-term exposure (30 min) to XH 100 μM.

	V_{max} (μmol mg prot^{-1} 10 min^{-1})	K_m (μM)	n
Control	63042 ± 15312	2867 ± 1646	12
XH (100 μM)	40322 ± 8715*	2622 ± 1365	12

Data are arithmetic mean ± SEM.
*$P < 0.05$ significantly different from control (Student's t test).

cellular deprivation. A further confirmation of this hypothesis was the observation that high glucose conditions were able to reverse the effect of XH in relation to cell viability and culture growth (Fig. 9C and D). However, low extracellular glucose conditions did not mimic, and high glucose conditions did not reverse, the effect of XH (5 μM) upon cell migration (Fig. 9 E and F). These findings suggest that the antimigratory effect of XH is not dependent on inhibition of glucose entry and thus is not related to glucose cellular deprivation.

Discussion

Uptake of glucose by term trophoblasts and its modulation by xenobiotics has been previously studied by our group. We have verified that dietary compounds such as RESV, XH, EGCG, QUE and TEO decreased ^{3}H-DG uptake by a human choriocarcinoma cell line (Araújo *et al.*, 2008). In the present study, we intended to extend that previous investigation, by characterizing glucose uptake and its modulation by endo and xenobiotics in first-trimester EVTs. The compounds tested included the serotonergic agents serotonin and FLUOX, the hormones DEXA and MELAT, several dietary compounds (polyphenols: QUE (a flavonol), EGCG (a flavan-3-ol), XH (a chalchone) and RESV (a stilbene) and methylxanthines (CAF and TEO) and the drugs of abuse amphetamine, MDMA, nicotine, cocaine and ethanol.

The cell line HTR-8/SVneo has been established by transfecting normal first-trimester trophoblasts with a plasmid containing the gene for the simian virus 40 (SV40) large T antigen (Tag). This cell line retains the epithelial phenotype and the proliferative and invasive characteristics of EVTs, thus being an accepted *in vitro* model system for the study of EVTs characteristics (Graham *et al.*, 1993).

For glucose transport experiments, ^{3}H-DG was used. This compound is a glucose analog efficiently transported by GLUT family members, but a very poor SGLT substrate (Wright *et al.*, 2003), and is not metabolized by phosphoglucose isomerase (Dwarakanath, 2009). ^{3}H-DG uptake by HTR-8/SVneo cells was found to be time-dependent, saturable, inhibited by CYT B, PHT and PHZ, insulin-insensitive and sodium-independent. These characteristics led us to conclude that a GLUT transporter is the main mediator of DG uptake by HTR-8/SVneo cells, most probably GLUT1 since this transporter is highly expressed in this cell line (Belkacemi *et al.*, 2005) and it is known to be critical for glucose uptake in placenta throughout pregnancy (Illsley, 2000).

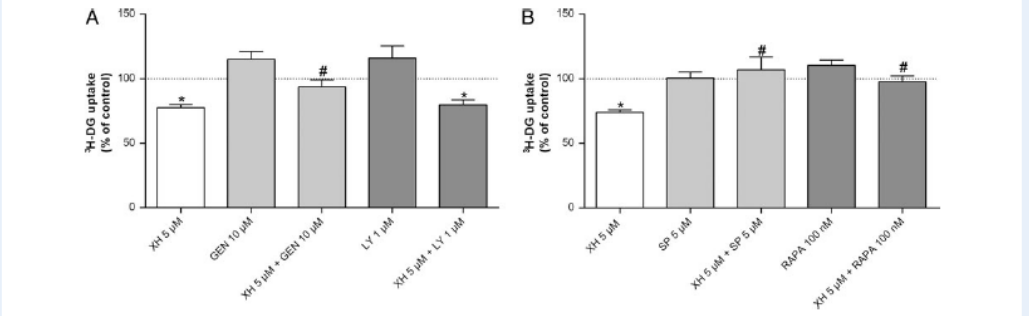

Figure 7 The inhibitory effect of long-term exposure to XH upon ^{3}H-DG uptake by HTR-8/SVneo cells involves mammalian target of rapamycin, tyrosine kinase and c-Jun N-terminal kinase signaling pathways. Cells were preincubated for 24 h in serum-free culture medium and then incubated with 10 nM ^{3}H-DG for 10 min, in the absence or presence of 5 µM XH ($n = 11–21$) and (**A**) genistein (GEN; 10 µM; $n = 12$) or LY 294002 (LY; 1 µM; $n = 9$); or (**B**) SP 600125 (SP; 5 µM; $n = 12$) or rapamycin (RAPA; 100 nM; $n = 9$). Shown are arithmetic means ± SEM. $^*P < 0.05$ significantly different from control (dashed line), $^\#P < 0.05$ significantly different from XH 5 µM (one-way analysis of variance followed by Bonferroni test).

A short-term exposure of HTR-8/SVneo cells to FLUOX (100–1000 µM) inhibited ^{3}H-DG uptake. FLUOX is an antidepressant drug that belongs to the selective serotonin reuptake inhibitors (SSRI) class. However, the effect of FLUOX upon ^{3}H-DG uptake does not appear to be related to its effect upon the serotonin transporter (SERT), as serotonin (100–1000 µM) did not alter ^{3}H-DG uptake. During pregnancy and in the immediate post-natal period, 10% of women present a major depressive disorder (Belik, 2008), and it is estimated that 2–3% of pregnant women receive SSRI antidepressive treatment (Belik, 2008). We show here that administration of these drugs can impact EVTs nutrition.

Additionally, a short-term exposure of HTR-8/SVneo cells to the synthetic glucocorticoid DEXA (100–1000 µM) was also found to induce a concentration-dependent inhibition of ^{3}H-DG uptake. An inhibitory effect of DEXA in relation to glucose uptake has previously been described in other cell types, namely in 3T3–L1 adipocytes (Houstis *et al.*, 2006), associated with a repression of GLUT4 expression (Sangeetha *et al.*, 2013), and in rat PC12 chromaffin tumor cells, associated with a reduction in GLUT3 protein expression (Liu *et al.*, 2014). This is interesting, as DEXA administration during pregnancy is commonly used to induce fetal lung maturation (Brownfoot *et al.*, 2013). As recently reviewed by us (Correia-Branco *et al.*, 2015), fetal exposure to DEXA can have several developmental programing adverse effects upon fetal health, including development of insulin resistance in adulthood. In this context, our results may provide a mechanism linking fetal exposure to DEXA to the developmental programing of insulin resistance.

Finally, a short-term exposure of HTR-8/SVneo cells to the polyphenols QUE (30–1000 µM), EGCG (30–1000 µM), XH (1–500 µM) and RESV (1–500 µM) and to the methylxanthines CAF (100–1000 µM) and TEO (100–1000 µM) induced a concentration-dependent inhibition of ^{3}H-DG uptake. These results go in line with previous studies showing a glucose uptake inhibitory effect of these compounds in other cell lines. For instance, our group found an inhibitory effect of these polyphenols upon ^{3}H-DG uptake by a human term trophoblast cell line (BeWo cells), although the methylxanthines were devoid of effect (Araújo *et al.*, 2008). Moreover, QUE and EGCG were found to reduce glucose uptake by MCF7 breast cancer cells (Moreira *et al.*, 2013), and QUE reduced glucose uptake by another breast cancer cell line (MDA-MB-231 cells), associated with a decrease in cell membrane translocation of GLUT4 (Wang and Yang, 2014). Also, an inhibitory effect of QUE and EGCG upon glucose uptake and GLUT4 expression was observed in a mouse preadipocyte cell line, MC3T3-G2/PA6 (Nomura *et al.*, 2008) and in isolated rat adipocyte cells (Strobel *et al.*, 2005). As for RESV, it was reported to inhibit glucose uptake in Lewis lung carcinoma, HT-29 colon, and in T47D breast cancer cells, but the identity of the transporter(s) involved was not reported (Jung *et al.*, 2013). As for the methylxanthines, CAF and TEO were previously found to inhibit glucose uptake by noncompetitive inhibition of GLUT1 in unsealed erythrocyte ghosts from washed red blood cells (Ojeda *et al.*, 2012) and CAF inhibited glucose uptake and suppressed insulin-induced GLUT4 translocation in mouse preadipose MC-3T3-G2/PA6 cells (Akiba *et al.*, 2004).

Among all the compounds tested, XH was the most potent inhibitor of ^{3}H-DG uptake by HTR-8/SVneo cells. XH is a structurally simple prenylated chalcone that occurs only in the hop plant, *Humulus lupulus L.* (Cannabaceae), where it is the principal prenylflavonoid of the female inflorescences (usually referred to as 'hops' or 'hop cones'), and is used to add bitterness and flavor to beer (Stevens and Page, 2004). The effect of this chalcone was very potent: exposure to XH inhibited ^{3}H-DG uptake (IC_{50} of 3.55 µM) in a concentration-dependent manner, and it behaved as a noncompetitive inhibitor (decreasing V_{max} while not affecting the *Km* of ^{3}H-DG uptake).

The effect of XH was consistent as long-term (24 h) exposure of HTR-8/SVneo cells to this chalcone was also able to induce a reduction in ^{3}H-DG uptake in a noncompetitive manner. Interestingly enough, this effect was associated neither with a decrease in GLUT1 mRNA expression levels nor with a decrease in GLUT1 protein levels, suggesting that the reduction of ^{3}H-DG transport induced by XH is related to changes in the intrinsic activity of the GLUT1 transporter. Moreover, we could

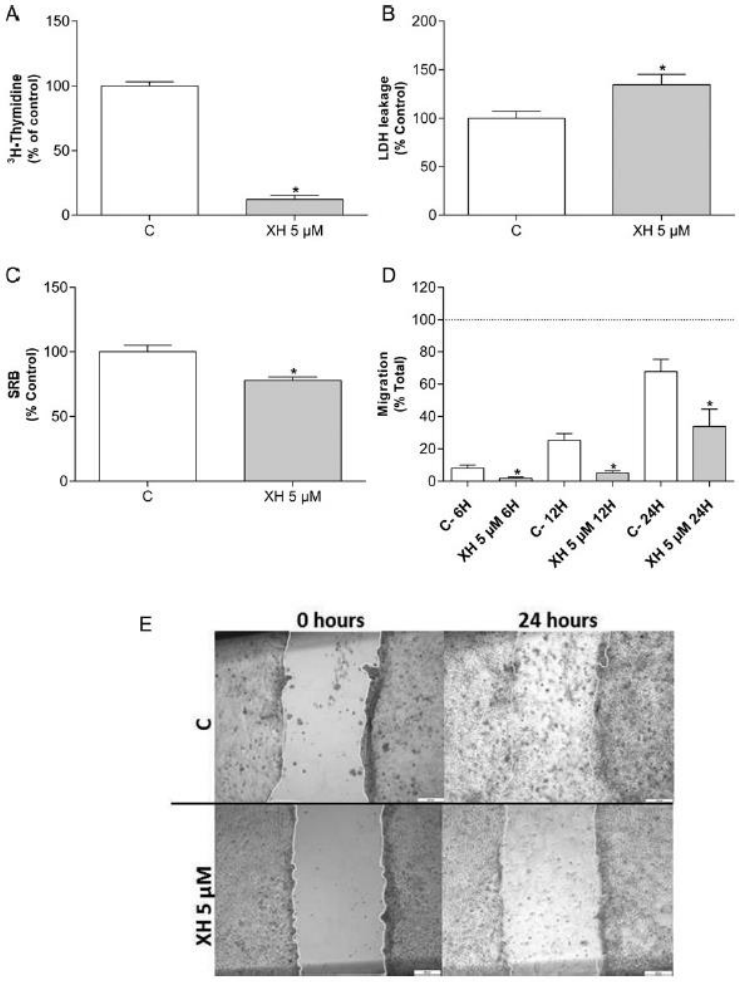

Figure 8 XH presents antiproliferative, cytotoxic and antimigratory effect on HTR-8/SVneo cells. Cells were exposed to 5 µM XH or the respective solvent (control, C) at 37°C, pH 7.4, for 24 h in serum-free culture medium. The effect of XH was tested in relation to: (**A**) cell proliferation rates ($n = 8$); (**B**) cell viability ($n = 8$); (**C**) culture growth ($n = 8$) and (**D**) cell migration at 6 h ($n = 8$), 12 h ($n = 8$) and 24 h ($n = 12$). (**E**) Representative image of cell migration determination by wound healing assay. Cell monolayers were scratched with a pipette tip and then treated for 24 h with XH (or the respective vehicle). Images shown were obtained at 0 and 24 h after scratching. Scale bars: 200 µm. Shown are arithmetic means ± SEM. *$P < 0.05$ significantly different from control (dashed line) (Student's *t* test).

conclude that the inhibitory effect of XH upon ^{3}H-DG uptake involves the intracellular signaling pathways mTOR, TK and JNK, but not PI3K. Interestingly enough, in a recent study in brown adipose tissue, it was shown that GLUT1 transcription and de novo synthesis of GLUT1 are mediated by mTOR and independent of the classical insulin–PI3K pathway (Olsen *et al.*, 2014). Also, it has been described that several TK inhibitors, either synthetic (such as the tyrphostins) or natural products of the family of flavones and isoflavones (including genistein), are able to impair GLUT1 activity, apparently by competition with the ATP binding site of GLUT1 (Vera *et al.*, 2001).

In the last part of this work, the relationship between inhibition of glucose uptake by XH and the placentation process was investigated.

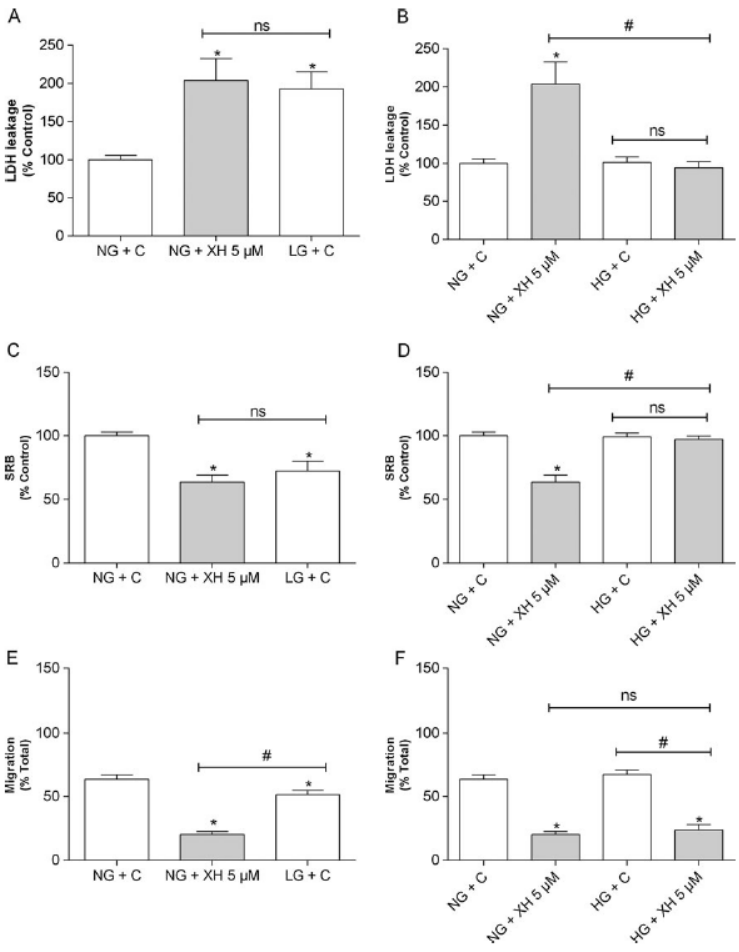

Figure 9 The antiproliferative and cytotoxic effect, but not the antimigratory effect of XH is dependent on inhibition of glucose uptake by HTR-8/SVneo cells. Cells were exposed to 5 µM XH or the respective solvent for 24 h in low glucose conditions (LG+C, 1 mM), normal glucose conditions (NG+C, 11 mM) or high glucose conditions (HG+C, 20 mM). Low extracellular glucose conditions mimic the effect of 5 µM XH upon cell viability (Panel A; $n = 12$) and culture growth (Panel C; $n = 12$), but not cell migration (Panel E; $n = 15–20$). High glucose conditions revert the effect of 5 µM XH upon cell viability (Panel B; $n = 12$) and culture growth (Panel D; $n = 12$), but not cell migration (Panel F; $n = 15–20$). Shown are arithmetic means ± SEM. $^{*}P < 0.05$ significantly different from control (NG+C); ns = not significantly different from each other; $^{\#}P < 0.05$ significantly different from each other (Student's *t* test).

The long-term (24 h) exposure of the cells to this dietary polyphenol caused a significant decrease in cell viability, culture growth, proliferation and migration. By showing that low extracellular glucose mimics the effect of XH upon cell viability and culture growth, and that high extracellular glucose reverses these effects of XH, we can conclude that the antiproliferative and cytotoxic effects of XH are dependent upon glucose deprivation of HTR-8/SVneo cells. However, these findings do not apply to the antimigratory effect of XH, suggesting that this effect is not dependent upon glucose deprivation of HTR-8/SVneo cells.

It is interesting to observe that XH, a polyphenol with a chemopreventive effect (Gerhauser *et al.*, 2002; Vene *et al.*, 2012; Wyns *et al.*, 2012; Blanquer-Rossello *et al.*, 2013; Kim *et al.*, 2013; Tronina *et al.*, 2013), shows a similar effect in relation to EVT proliferation, culture growth, cell viability and migration. Also, changes in viability, culture growth, proliferation and migration after exposure of HTR-8/SVneo cells to XH are

similar to the phenotype observed in insufficient EVTs from pregnancy-related pathologies such as FGR. Indeed, EVT insufficiency and inadequate placentation, characterized by shallow cellular viability, proliferation, migration, invasion and angiogenesis, is believed to be an underlying mechanism of FGR (Fisher, 2004). As such, exposing HTR-8/SVneo cells to XH could provide a means to develop an experimental cell model of first-trimester insufficient EVTs, which is presently still lacking.

Inhibition of glucose uptake is expected to be associated with a consequent reduction in glucose metabolism and thus in lactate production. However, contrary to our expectations, exposure of cells to XH either for short-term (30 min) or for long term (24 h) induced a very marked increase in the amount of extracellular lactate levels. We hypothesized that this observation is related to an inhibitory effect of XH upon MCT1-mediated reuptake of lactate. The observation that XH was indeed able to reduce MCT1-mediated uptake of ^{14}C-BT supported our hypothesis. We then conclude that XH inhibits MCT1-mediated lactate reuptake by HTR-8/SVneo cells. By inhibiting lactate reuptake in these cells, XH may also starve cells of lactate, which thus further contributes to cell death.

In conclusion, XH seems to be a potent noncompetitive inhibitor of glucose uptake by HTR-8/SVneo cells, either after short- or long-term exposure, and this effect of XH seems to involve mTOR, TK and JNK intracellular pathways. In addition, the antiproliferative and cytotoxic effects of XH in these cells are related to cellular glucose deprivation, as they were mimicked by low extracellular glucose conditions and reversed by high glucose extracellular conditions. Furthermore, XH increases extracellular lactate levels, associated with an inhibition in MCT1-mediated lactate cellular reuptake, pointing to an alteration of both glucose and lactate handling by these cells. As such, this study contributes to a deeper knowledge of the impact of XH upon the placentation process and consequently the nefarious impact upon fetal and maternal health, which can have long-term consequences upon the developmental programing effects of disease (Correia-Branco *et al.*, 2015).

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

A.C.-B. contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and drafting of the article. C.F.A., J.R.A., J.T.G. and A.F. contributed to the acquisition of data. E.K. contributed to conception and design of the study, analysis and interpretation of data and revised the article for important intellectual content. F.M. contributed to conception and design of the study, analysis and interpretation of data, revised the article for important intellectual content and gave the final approval of the version to be published.

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Conflict of interest

None declared.

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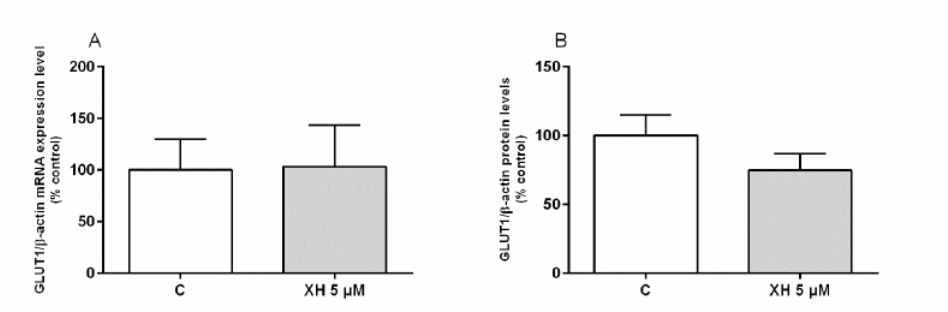
Supplementary Figure S1 Lack of effect of xanthohumol upon GLUT1 mRNA and protein levels.

Cells were incubated for 24 h in serum-free culture medium at 37°C, pH 7.4, in the presence of 5 µM xanthohumol (XH; n=5-6) or the respective solvent (control, C; n=6). The effect of XH 5 was analyzed in relation to (A) GLUT1 mRNA and (B) protein levels. Shown are arithmetic means±SEM.



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Involvement of mTOR, JNK and PI3K in the negative effect of ethanol and metformin on the human first-trimester extravillous trophoblast HTR-8/SVneo cell line

(Accepted article)

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23 Abstract

24 Our aim was to investigate the effect of two xenobiotics to which pregnant woman may be
25 exposed, the drug of abuse ethanol (EtOH) (and its metabolite acetaldehyde (ACA)) and the
26 therapeutic agent metformin (METF), on placentation-related processes in an extravillous
27 trophoblastic (EVTs) cell line (HTR-8/SVneo cells). EtOH, ACA and METF (24h) significantly
28 reduced cell proliferation rates, culture growth, viability and migratory capacity of HTR-8/SVneo
29 cells. Moreover, both EtOH (100 μM) and METF (1 mM) increased the apoptosis index and
30 inhibited ^{3}H-deoxy-D-glucose (^{3}H-DG) and ^{3}H-folic acid (^{3}H-FA) uptake. mTOR, JNK and PI3K
31 intracellular signaling pathways were involved in the effect of EtOH upon ^{3}H-FA uptake and in
32 the effect of METF upon cell viability, and mTOR and JNK in the effect of EtOH upon cell viability
33 and ^{3}H-DG uptake.
34 We show that EtOH and METF have a detrimental effect in placentation-related processes of
35 HTR-8/SVneo cells. Moreover, mTOR, JNK and PI3K appear to mediate some of these negative
36 effects.

37

38

39

40

41 **Keywords**: mTOR, JNK, PI3K, ethanol, metformin; trophoblasts

42

43 **Abbreviations:**

44 ACA, acetaldehyde; DG, D(+)-glucose; EtOH, ethanol; EVT, extravillous trophoblast; FA, folic
45 acid; FAS, fetal alcohol syndrome; FASD, fetal alcohol spectrum disorders; FGR, fetal growth
46 restriction; FR, folate receptor; GLU, glucose; GLUT, glucose transporter; GF-HBS, glucose-free-
47 HEPES-buffered saline; JNK, c-Jun N-terminal kinases; LDH, lactate dehydrogenase; METF,
48 metformin hydrochloride, 1,1-dimethylbiguanide hydrochloride; mTOR, mammalian target of
49 rapamycin; PCFT, proton-coupled folate transporter; PI3K, phosphatidylinositol-4,5-
50 bisphosphate 3-kinase; RAPA, rapamycin; RFC1, reduced folate carrier 1; SGLT, sodium-
51 dependent glucose co-transporters; SRB, sulphorhodamine B; TUNEL, terminal deoxynucleotidyl
52 transferase mediated deoxyuridine triphosphate nick-end labeling
53

1. Introduction

The placenta constitutes a boundary between the fetal and maternal environments, being involved in the exchange of nutrients and waste products between the mother and the fetus (Cross, 1998; Fowden et al., 2008). A compromised uterine blood flow and placental formation and development - placentation - causes reduced fetal nutrient uptake and fetal hypoxia, as observed in pathologies such as fetal growth restriction and preeclampsia (James et al., 2012).

Pregnant women are frequently exposed to several xenobiotics due to lifestyle factors such as diet, smoking, drug abuse, alcohol consumption or therapeutic drugs. This work focused in two xenobiotics, ethanol (EtOH) and metformin (METF). EtOH was chosen because previous experiments from our group showed a detrimental effect of this compound on term trophoblasts (Keating et al. 2009), and METF was chosen because it possesses an anticancer effect, thus interfering with cell characteristics such as proliferation and apoptosis (Daugan et al. 2016).

EtOH is one of the most frequently used drugs worldwide (Halsted *et al.*, 2002), and although most women avoid alcohol during pregnancy, many of them begin to reduce consumption only upon pregnancy recognition (Bhuvaneswar, 2007; Skagerström 2013). In the U.S., about 10% of pregnant women report alcohol use (Schuchat, 2017). Alcohol consumption during pregnancy can lead to brain, craniofacial, cardiovascular and limb defects collectively termed Fetal Alcohol Spectrum Disorders (Abel, 1984; Schuchat, 2017).

METF is a biguanide agent that reduces hyperglycaemia (Kirpichnikov et al., 2002; Rowan *et al.*, 2011). METF is widely used as first-line treatment in type 2 diabetes and polycystic ovary syndrome (PCOS) (Charles et al., 2006) and is becoming increasingly accepted as an alternative to insulin during pregnancy, for the management of type 2 and gestational diabetes and PCOS (Rowan et al. 2008; Inzucchi et al., 2012; Qaseem et al., 2012; Ainuddin et al., 2015). Considering that 1 to 14% of all pregnancies are affected by gestational diabetes (with prevalence varying with population specific factors such as genetics, environment and screening and diagnostic methods), METF exposure during pregnancy is gaining an expressive importance (Sivalingam, 2014; Erem, 2015). Nonetheless, the effect of METF on the placenta and its long-term effect on fetal physiology has not been clearly elucidated (Rowan et al., 2008; Ainuddin et al., 2015).

We aimed to investigate the influence of EtOH - and its metabolite acetaldehyde (ACA) - and METF) on placentation-related processes (cell viability and proliferation, culture growth, migratory capacity and apoptosis index). Also, we intended to study the impact of EtOH, ACA and METF upon nutrient (glucose (GLU) and folic acid (FA)) uptake into extravillous trophoblast

(EVT) cells, since an adequate nutrition of EVT itself is critical for the normal process of placentation. For this, we used a human first-trimester extravillous trophoblasts (EVT) cell line (HTR-8/SVneo cells). EVTs are fully specialized trophoblasts displaying an invasive and proliferative phenotype, and they are the key cell type involved in the placentation process (Ji et al., 2013).

Moreover, we intended to investigate the putative involvement of mammalian target of rapamycin (mTOR), c-Jun N-terminal kinases (JNK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) intracellular signaling pathways in these effects. PI3K and mTOR are major signaling components that play a crucial role in growth factor-mediated first-trimester EVT cell migration (Qiu et al., 2004). More specifically, several growth factors are known to increase trophoblast motility by inducing activation of PI3K and consequently by downstream activating Akt and mTOR (Pollheimer and Knofler, 2005). Of note, mTOR signaling is influenced by a large number of upstream regulators such as amino acids, growth factors, and free fatty acids (Jansson et al., 2012; Dimasuay et al., 2016). Moreover, JNK pathway regulates migration in different cell types using multiple molecular mechanisms (Pollheimer and Knofler, 2005).

2. Material and Methods

2.1. Materials

^{3}H-2-deoxy-D-glucose (2-[1,2-^{3}H(N)]-deoxy-D-glucose; ^{3}H-DG; specific activity 50 Ci/mmol), ^{3}H-FA (folic acid, [3,5,7,9-^{3}H]-FA sodium salt; specific activity 40.0 Ci/mmol), (American Radiolabeled Chemicals Inc., St. Louis, MO, USA); ^{3}H-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH, Freiburg, Germany); acetaldehyde (ACA), antibiotic/antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B), HEPES-NaOH (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid), LY294002 hydrochloride, magnesium sulfate ($MgSO_4$), MES (N-2-hydromorfolin-ethanesulphonic acid), metformin hydrochloride (1,1-dimethylbiguanide hydrochloride; METF), NADH (reduced nicotinamide adenine dinucleotide), p-formaldehyde, potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), rapamycin (RAPA), RPMI 1640 medium, sodium citrate tribasic dehydrate, sodium chloride (NaCl), SP600125, sulphorhodamine B (SRB), trichloroacetic acid (TCA), triton X-100 and trypsin-EDTA solution (Sigma, St. Louis, MO, USA). Fetal bovine serum (Gibco, Life Technologies Corporation, CA, USA), D(+)-glucose (DG), DMSO

(dimethylsulphoxide), Tris (tris-(hydroxymethyl)-aminomethane hydrochloride), Triton X-100 (Merck, Dramstad, Germany).

The drugs to be tested were dissolved in water (ACA, DG, METF, NADH) or DMSO (LY294002, RAPA, SP600125). The final concentration of these solvents was 1% (v/v) in the culture media. Controls for these compounds were run in the presence of the respective solvents. Stock solutions (LY 294002 10 Mm, RAPA 1 mM and SP 600125 10 mM) were stored at -20°C, unless otherwise stated. The tested concentrations of EtOH (0.1-100 μM), ACA (0.1-100 μM) and METF (0.01-1 mM) were chosen based on previous works of our group (Correia-Branco et al., 2015; in press).

2.2. Human first trimester EVT cell culture (HTR-8/SVneo cells)

HTR-8/SVneo cells were generously donated by Dr. Charles H. Graham (Department of Anatomy & Cell Biology, Queen's University, Kingston, Canada) and were used between passage number 77 and 92. Cells were maintained in a humidified atmosphere of 5% CO_2 – 95% air and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution. Culture medium was changed every 2-3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.05% trypsin-EDTA, 5 min, 37°C), split 1:6 ratio and sub-cultured in plastic culture dishes (21 cm^2; Ø 60 mm TPP®, Trasadingen, Switerzland). For transport experiments and for quantification of cell viability, proliferation, culture growth and migration, HTR-8/SVneo cells were seeded on 24-well plastic cell culture dishes (2 cm^2; Ø 16 mm; TPP®), and were used after 9-14 days in culture (90-100% confluence). For determination of apoptosis index, cells were seeded on glass coverslips on 24-well plastic cell culture dishes (2 cm^2; Ø 16 mm; TPP®), and were used after 2 days in culture (20% confluence). In all experiments, the cell medium was made free of fetal calf serum during the last 24h.

2.3. Determination of cell viability

The long-term (24h) effect of compounds upon cellular viability was determined by quantification of extracellular activity of LDH, as previously described (Bergmeyer and Bernt, 1974; Correia-Branco et al., 2015).

2.4. Determination of cell proliferation rates

The long-term (24h) effect of compounds upon cellular proliferation rates was determined with the ^{3}H-thymidine incorporation assay, as previously described (Correia-Branco et al., 2015).

158

159 *2.5. Determination of culture growth*

160 The long-term (24h) effect of compounds on culture growth was determined by the SRB assay,
161 as described elsewhere (Correia-Branco et al., 2015).

162

163 *2.6. Determination of migration rates*

164 The long-term (24h) effect of compounds on cell migration rates were determined by the in
165 vitro wound healing assay, as previously described (Correia-Branco et al., 2015). Images were
166 obtained at 0 and 24h of treatment and quantification was performed by using ImageJ software
167 (Reinhart-King, 2008; Negrão et al., 2013).

168

169 *2.7. Determination of apoptosis index (TUNEL assay)*

170 The long-term (24h) effect of compounds on the apoptosis index was determined by the TUNEL
171 (terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick-end labeling)
172 assay and was executed using the In Situ Cell Death Detection kit (Roche Diagnostics,
173 Mannheim, Germany) according to the manufacturer's instructions and as described elsewhere
174 (Correia-Branco et al., 2015). Apoptosis index was calculated as the number of apoptotic cells
175 (in % of control).

176

177 *2.8. Transport studies*

178 For GLU transport experiments, ^{3}H-DG was used. DG is a GLU analog efficiently transported by
179 GLUT family members, but a very poor SGLT substrate (Wright *et al.*, 2003), and is not
180 metabolized by phosphoglucose isomerase (Dwarakanath, 2009).
181 ^{3}H-DG uptake experiments were performed in buffer with the following composition (in mM):
182 20 HEPES-NaOH, 5 KCl, 140 NaCl, 2.5 $MgSO_4$, 1 $CaCl_2$ and pH 7.4. ^{3}H-FA uptake experiments
183 were performed in buffer with the following composition (in mM): 12.5 HEPES-NaOH, 12.5 MES,
184 4.8 KCl, 125 NaCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 5.6 glucose and pH 5.5.
185 After an initial preincubation of the cell monolayers for 20 min in buffer at 37°C, uptake was
186 initiated by the incubation of 0.2 ml buffer at 37°C containing 10 nM ^{3}H-DG or 20 nM ^{3}H-FA.
187 Incubation was stopped after 10 min (^{3}H-DG) or 6 min (^{3}H-FA). Previous experiments from our
188 group have shown that ^{3}H-DG and ^{3}H-FA uptake by HTR-8/SVneo cells were linear with time up
189 to the tenth (Correia-Branco *et al.*, 2015) and sixth (Correia-Branco *et al.*, submitted) min of
190 incubation, respectively. Radioactivity in the cells was measured by liquid scintillation counting.

In order to test the long-term effect of compounds, cells were exposed to the compounds for 24h and during the preincubation and incubations periods (corresponding to a 30 min or 26 min exposure time, for ^{3}H-DG or ^{3}H-FA experiments, respectively).

2.9. Protein determination

The protein content of cell monolayers was determined as described by Bradford (Bradford, 1976), using human serum albumin as standard.

2.10. Calculations and Statistics

Arithmetic means are given with SEM. *n* represents the number of replicates of at least two different experiments. Statistical significance of the difference between two groups was evaluated by the Student's t-test and statistical significance of the difference between three or more groups was evaluated by one-way ANOVA test, followed by Tukey's test. Differences were considered to be significant when $P< 0.05$.

3. Results

3.1. Effect of EtOH, ACA and METF on placentation-related processes

We determined the effect of a 24h-exposure to EtOH, ACA and METF on cell proliferation rates, cell viability, culture growth, migration capacity, apoptosis index and nutrient (^{3}H-DG (10 nM) and ^{3}H-FA (20 nM)) uptake by HTR-8/SVneo cells.

3.1.1. Effect of EtOH and ACA

We verified that EtOH and ACA decreased cell proliferation rates in a concentration-dependent manner (Fig. 1A). Moreover, both EtOH (100 µM) and ACA (100 µM) reduced cell viability (Fig. 1B), cell culture growth (Fig. 1C) and cell migration (Fig. 1D; Fig. S1). EtOH (100 µM) was also found to increase apoptosis index (Fig. 1E) and to concentration-dependently (1-100 µM) reduce both ^{3}H-DG (10 nM) (Fig. 2A) and ^{3}H-FA (20 nM) (Fig. 2B) uptake by HTR-8/SVneo cells. So, both EtOH and ACA present deleterious effects in several parameters related to placentation.

226 *3.1.2. Effect of METF*

227 As observed by Fig. 3, METF (1 mM) significantly reduced cell proliferation rates (Fig. 3A), cell
228 viability (Fig. 3B), cell culture growth (Fig. 3C), cell migration (Fig. 3D; Fig. S1) and increased the
229 apoptosis index (Fig. 3E). Additionally, METF (0.01-1 mM) concentration-dependently reduced
230 both ^{3}H-DG (Fig. 4A) and ^{3}H-FA (Fig. 4B) uptake by HTR-8/SVneo cells. A lower concentration of
231 METF (0.01 mM), alone or in combination with hyperglycemia (20 mM glucose), was also able
232 to cause a significant decrease in cell viability and culture growth, but had no effect upon cell
233 migration (data not shown). METF thus presents deleterious effects in several parameters
234 related to placentation.
235
236 *3.2. Putative involvement of mTOR, c-JNK and PI3K intracellular signaling pathways on the effect*
237 *of EtOH and METF on cell viability, culture growth and nutrient uptake*
238 We tested the involvement of mTOR, JNK and PI3K on the effect of EtOH (100 μM) and METF (1
239 mM) upon cell viability, culture growth and nutrient (^{3}H-DG (10 nM) and ^{3}H-FA (20 nM)) uptake,
240 by using specific inhibitors of these intracellular pathways.
241
242 *3.2.1. EtOH*
243 The effect of EtOH (100 μM) upon cell viability (Fig. 5A) and ^{3}H-DG uptake (Fig. 5C) was reversed
244 by the mTOR inhibitor RAPA (100 nM) and by the JNK inhibitor SP600125 (5 μM), but not by the
245 PI3K inhibitor LY294002 (1 μM). This indicates the involvement of mTOR and JNK, but not the
246 PI3K, intracellular signaling pathways in these effects. Furthermore, the effect of EtOH upon ^{3}H-
247 FA uptake was completely abolished by RAPA and reduced by SP600125 and by LY294002,
248 indicating the involvement of mTOR, JNK and PI3K intracellular signaling pathways in this effect
249 (Fig. 5D). In contrast, the involvement of mTOR, JNK and PI3K signaling intracellular pathways on
250 the effect of EtOH upon cell culture growth was excluded due to the lack of efficacy of the
251 respective inhibitors in inhibiting/abolishing the effect of EtOH (Fig. 5B).
252 Interestingly, RAPA (100 nM), SP600125 (5 μM) and LY294002 (1 μM) alone presented an
253 inhibitory effect upon cell culture growth, indicating the involvement of mTOR, JNK and PI3K
254 intracellular signaling pathways in cell culture growth in this cell line (Figs. 5B and 6B).
255
256 *3.2.2. METF*
257 The effect of METF (1 mM) upon cell viability was completely abolished by the inhibitors RAPA
258 (100 nM), SP600125 (5 μM) and LY294002 (1 μM), which indicates the involvement of the
259 mTOR, JNK and PI3K signaling intracellular pathways in this effect (Fig. 6A). On the contrary, the
260 effect of METF upon culture growth (Fig. 6B), ^{3}H-DG uptake (Fig. 6C) and ^{3}H-FA uptake (Fig. 6D)

was not affected by these compounds, excluding the involvement of these intracellular signaling pathways in the effects of METF.

4. Discussion

The aim of this work was to test the effect of the drug of abuse EtOH - and its metabolite ACA – and of the therapeutic drug METF on placentation-related processes in first-trimester EVTs. These compounds were chosen because they are used by some pregnant women, and, for EtOH, its harmful effects upon the fetus is well known. Acetaldehyde (ACA) is a metabolite of ETOH also found to be present in the placenta, amniotic fluid and fetal liver (Hard *et al.*, 2001) and has genotoxic effects in humans (Kayani and Parry, 2010) and in animal models (Sanchis et al., 1987). The concentrations of EtOH, ACA and METF used in this *in vitro* study fall within the blood levels found in exposed women. Indeed, EtOH was tested in concentrations (0.1-100 μM) below the maximum permissible blood EtOH levels in Portugal (0.05 g/dl; approximately 10 mM), ACA was tested in concentrations (0.1-100 μM) ranging from below to higher than the average peak blood concentration (26-43 μM) (Lui et al., 2014), and METF was tested in concentrations (0.01-1 mM) ranging from below to higher than the commonly found plasma levels (He and Wondisford, 2015).

Among the placentation-related processes, we determined the effect of EtOH and METF on the cellular uptake of two important nutrients, namely GLU and FA. GLU is a major substrate for fetal and placental energy metabolism. Because gluconeogenesis in the feto-placental unit is minimal (Magnusson et al., 2004), GLU supply from the maternal circulation is essential for placentation and normal fetal development and growth (Baumann et al., 2002; Carter, 2012; Illsley, 2000). Our group previously described that GLU uptake by HTR-8/SVneo cells seems to be GLUT1-mediated (Correia-Branco et al., 2015). FA is the parent structure of a large family of B-vitamin coenzymes known as folates (Lucock, 2000; Pitkin, 2007; Gong et al., 2016), involved in the synthesis of purine and pyrimidine precursors of nucleic acids, the metabolism of certain amino acids, and the initiation of mitochondrial protein synthesis (Lucock, 2000; Pitkin, 2007; Gong et al., 2016). FA is of major importance for normal fetal development, as a well-established association between maternal folate deficiency and low birth weight, increased risk of spontaneous abortion, and neural tube defects (e.g. spina bifida and anencephaly) is known to exist. Moreover, there is accumulating evidence that supplementation with FA during the periconceptional period reduces the incidence of low birth weight newborns and neural tube defects (Lucock, 2000; Pitkin, 2007; Gong et al., 2016). The human placenta expresses reduced

folate carrier (Prasad et al., 1995; Whetstine et al., 2002; Ganapathy et al., 2004; Zhao et al., 2011), folate receptor α and β (Ganapathy et al., 2004; Kamen and Smith, 2004; Zhao et al., 2011), Matherly and Goldman, 2003) and proton-coupled folate transporter (PCFT) (Zhao and Goldman, 2007; Zhao et al., 2011). A recent work by our group points to the involvement of PCFT in FA uptake by HTR-8/SVneo cells (Correia-Branco et al., in press). Despite the importance of both GLU and FA for the placentation process, GLU and FA homeostasis in first-trimester trophoblasts and the implications of these mechanisms in the process of placentation remains largely unexplored. Our results, showing a negative impact of EtOH and METF on the cellular uptake of both ^{3}H-DG and ^{3}H-FA, suggest that these compounds will interfere with the cellular handling of these nutrients. In this context, it would be interesting to determine the effect of EtOH and METF on the expression levels of GLUT1 and PCFT and on the cellular handling of these nutrients (eg. by determining glycolytic rates and dihydrofolate reductase activity).

The HTR-8/SVneo cell line displays an unlimited lifespan in culture and retain characteristics of invasive trophoblasts such as expression of cytokeratin 18 and some EVT-specific integrins (Graham et al., 1993; Knofler, 2010). This cell line is well accepted as a suitable *in vitro* model system for the study of first-trimester EVTs characteristics. A recent study showed that the HTR-8/SVneo cell line contains two mixed populations of cells, namely trophoblast and stromal/mesenchymal cells (Abou-Kheir et al., 2017). The fact that the HTR-8/SVneo cell line presents a mixed stromal/mesenchymal population of cells might reflect what undergoes *in vivo* during the placentation process. Indeed, at the tips of the anchoring villi, extravillous cytotrophoblasts (evCTB) undergo a transition from epithelial-to-mesenchymal phenotype, loosing epithelial markers and acquiring mesenchymal markers when they start to invade and migrate into the maternal endometrium and spiral arteries (Abou-Kheir et al. 2017).

Regarding EtOH, in a prospective study from the years 2000 to 2012 including 5,036 pregnant women, 55% reported alcohol use in the first trimester (Pryor et al., 2017), and 10% reported alcohol use during all the pregnancy period (Schuchat, 2017). Alcohol use during pregnancy is associated with increased maternal and (or) fetal morbidity and mortality, higher risk of low birth weight, preterm delivery and teratogenesis (Wright and Walker, 2001; Neri et al., 2015). Prenatal alcohol exposure can lead to alcohol-related birth defects such as spontaneous abortion, decreased immune function, attention problems, hearing impairment (Sundelin-Wahlsten *et al*., 2017) and fetal alcohol spectrum disorder (FASD) (Goodlett et al., 2005). Among children with FASD, a small population presents a specific set of anomalies (specific facial abnormalities, fetal growth retardation and significant neurodevelopmental impairment) known as fetal alcohol syndrome (FAS) (Goodlett et al., 2005; Krulewitch, 2005; Sundelin-Wahlsten et al., 2017). In this study, we observed that both EtOH and its metabolite

ACA presented a potent antiproliferative, cytotoxic and antimigratory effect in HTR-8/SVneo cells. Furthermore, EtOH markedly induced cellular apoptosis and concentration-dependently inhibited ^{3}H-DG and ^{3}H-FA uptake. Altogether, these results suggest that one possible mechanisms underlying EtOH harmfulness during pregnancy may be its potent negative effect upon placentation-related processes, including placental uptake of GLU and FA, with a consequent insufficient nutrient supply to the placenta and the fetus. In line with this observation, previous studies from our group demonstrated that exposure to EtOH reduced ^{3}H-DG uptake by primary cultures of human term cytotrophoblasts (Keating et al., 2008) and BeWo cells (Keating et al., 2009). In contrast, first trimester villous explants and the first trimester EVT cell line SGHPL4 treated for 24h with EtOH (20-40 mM) showed reduced proliferation and EtOH (40 mM) inhibited amino acid uptake by EVTs explants, but no effect on either cell invasion and cell apoptosis index in both cell models was found (Lui et al., 2014). Moreover, a reduction in EVTs explants proliferation was observed after exposure to ACA (40 µM), but no effect was found in the other parameters tested (Lui et al., 2014). Of note, distinct concentrations of EtOH (100 µM and 40 mM) and ACA (100 µM and 40 µM) were used in the present study and in the study of Lui et al., respectively, which may well explain the contrasting effects found.

METF is widely used as first-line treatment in type 2 diabetes and PCOS (Charles et al., 2006) and is becoming increasingly accepted as an alternative to insulin during pregnancy, for the management of type 2 and gestational diabetes and PCOS (Rowan et al., 2008; Inzucchi et al., 2012; Qaseem et al., 2012; Ainuddin et al., 2015). In the present study, we show that METF possesses an antiproliferative, cytotoxic, antimigratory and proapoptotic effect; moreover, it also concentration-dependently inhibits ^{3}H-DG and ^{3}H-FA uptake by HTR-8/SVneo cells. These results point to the harmfulness of this therapeutic drug upon the placentation process and to insufficient nutrient supply to the placenta and the fetus.

In the last part of this work, we investigated the putative involvement of PI3K, mTOR and JNK intracellular signaling pathways upon the effect of EtOH and METF. By using specific inhibitors of these intracellular signaling pathways, we conclude that the effect of EtOH upon ^{3}H-FA uptake involves mTOR, JNK and PI3K, and that its effect upon cell viability and ^{3}H-DG uptake involves mTOR and JNK. Moreover, mTOR, JNK and PI3K are also involved in the cytotoxic effect of METF. Also, activation of mTOR, JNK and PI3K intracellular signaling pathways seems to be involved in cell culture growth in this cell line.

We have recently shown that inhibition of ^{3}H-DG (Correia-Branco et al., 2015) and ^{14}C-arachidonic acid (Correia-Branco et al., in press) uptake by the dietary polyphenol xanthohumol in this same cell line also involves JNK and mTOR intracellular pathways, and several studies point to the crucial involvement of mTOR and JNK pathways upon the process of placentation.

Indeed, RAPA, which specifically blocks mTOR, decreases migration of HTR-8/SVneo cells (Qiu et al., 2004), and mTOR appears to be essential for growth and proliferation of early mouse embryos and embryonic stem cells (Gangloff et al., 2004; Murakami et al., 2004; Wen et al. 2005). Also, activation of JNK signaling pathway results in changes in migration and invasion of HTR-8/SVneo cells (*Liu et al.,* 2016). A recent study showed a reduction in placental mTOR in FGR term placentas, in association with increased autophagic vacuoles, thus pointing to a reciprocal regulation between mTOR signaling and FGR (Zhang et al., 2017). As such, downregulation of mTOR by METF and EtOH may provide an important clue for the mechanism involved in the negative effect of these xenobiotics upon placentation *in vivo*. Moreover, PI3K also appears to be involved in the effect of METF and EtOH. This is most interesting, as PI3K is an up-regulator of mTOR. Because AKT upstream signaling PI3K and the downstream mTOR are involved in the effect of METF and EtOH, we can hypothesize that AKT plays also a critical role in the harmful effect of METF and EtOH in placentation-related processes.

In summary, our results show a detrimental effect of the drugs of abuse EtOH and of the therapeutic agent METF in placentation-related processes in EVTs. We also demonstrate that mTOR, JNK and PI3K intracellular signaling pathways are involved in the negative effect of EtOH and METF upon placentation-related processes such as cell viability and GLU and FA uptake.

5. Declaration of interest

Conflicts of interest: none.

Declaration of author's roles

A Correia-Branco contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and drafting of the article.

E Keating contributed to conception and design of the study and to data analyses and discussion, and revised the article for important intellectual content.

F Martel contributed to conception and design of the study, and to data analyses and discussion, revised the article for important intellectual content and gave the final approval of the version to be published.

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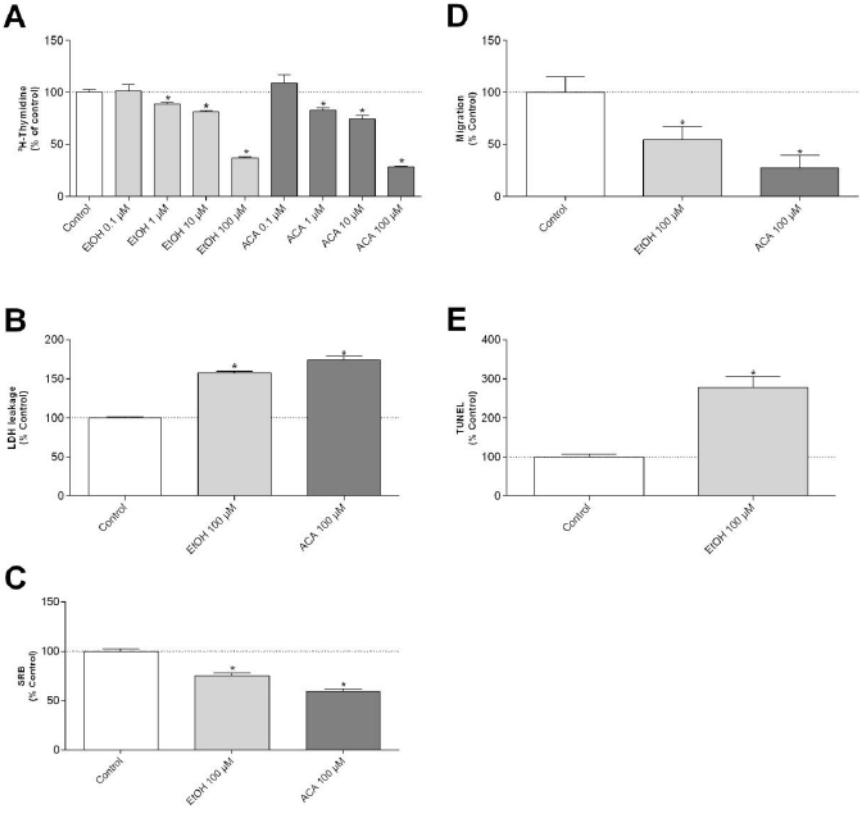
556

Figure legends

Figure 1 Characterization of the effect of ethanol and acetaldehyde upon cell proliferation rates, cell viability, culture growth, cell migration and apoptosis index of HTR-8/SVneo cells.

A: Cells were exposed for 24h to increasing concentrations of ethanol (EtOH 0.1-100 µM; n=4-8), acetaldehyde (ACA 0.1-100 µM; n=4-8) or the respective solvent (control, indicated by the dashed line; n=16) and the effect upon cell proliferation rate was evaluated. B-D: Cells were exposed for 24h to ethanol (EtOH 100 µM; n=6-14), acetaldehyde (ACA 100 µM; n=6-14) or the respective solvent (control, indicated by the dashed line; n=6–17) and the effect upon cell viability (panel B), culture growth (panel C) and cell migration (panel D) was evaluated. E: Cells were exposed for 24h to ethanol (EtOH 100 µM; n=22) or the respective solvent (control, indicated by the dashed line; n=22) and the effect upon cell apoptosis index (panel E) was evaluated. Shown are arithmetic means±SEM. * P<0.05 significantly different from control; A-D: one-way analysis of variance followed by Tukey's test; E: Student's t test.



572 **Figure 2** Characterization of the effect of ethanol upon nutrient (^{3}H-DG and ^{3}H-FA)
573 uptake by HTR-8/SVneo cells.

574 Cells were exposed for 24h to ethanol (EtOH 1-100 µM; n=10-14) or the respective solvent
575 (control, indicated by the dashed line; n=12) and the effect upon uptake of 10 nM ^{3}H-DG (panel
576 A) or 20 nM ^{3}H-FA (panel B) was evaluated. Shown are arithmetic means±SEM. * P<0.05
577 significantly different from control (one-way analysis of variance followed by Tukey's test).
578

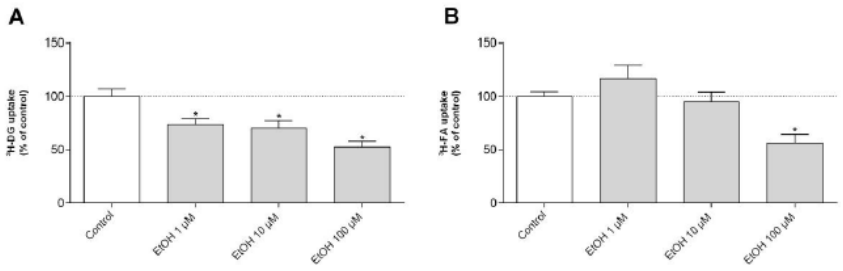
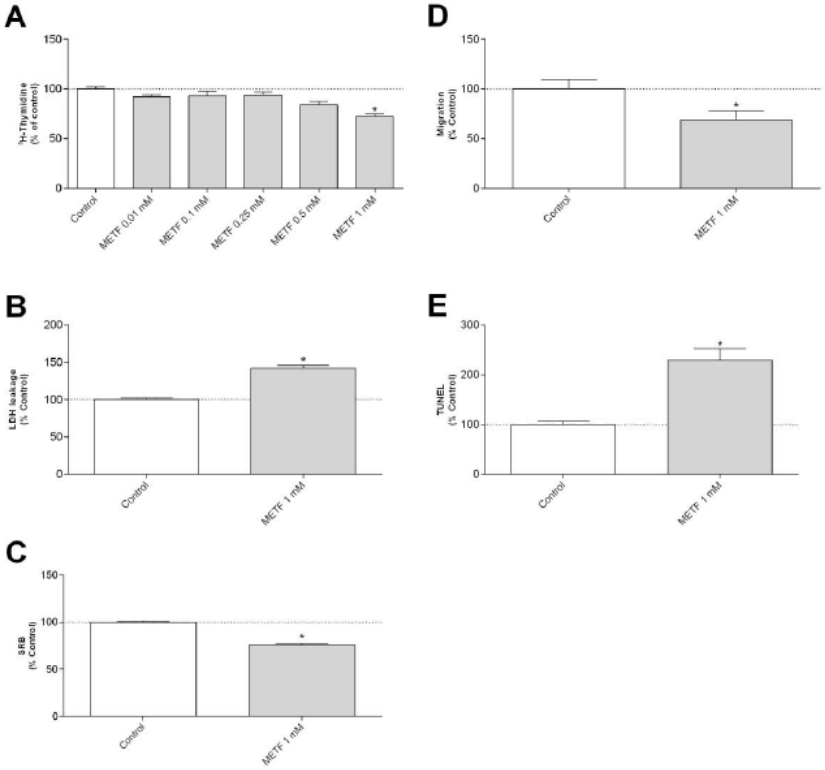


Figure 3 Characterization of the effect of metformin upon cell proliferation rates, cell viability, culture growth, cell migration and apoptosis index of HTR-8/SVneo cells.

A: Cells were exposed for 24h to increasing concentrations of metformin (METF 0.01-1 mM; n=3-12) or the respective solvent (control, indicated by the dashed line; n=11) and the effect upon cell proliferation rate was evaluated. B-E: Cells were exposed for 24h to metformin (METF 1 mM; n=16-22) or the respective solvent (control, indicated by the dashed line; n=14–22) and the effect upon cell viability (panel B), culture growth (panel C), cell migration (panel D) and cell apoptosis index (panel E) was evaluated. Shown are arithmetic means±SEM. * $P<0.05$ significantly different from control; A: one-way analysis of variance followed by Tukey's test; B-E: Student's t test.



590 **Figure 4** Characterization of the effect of metformin upon nutrient (^{3}H-DG and ^{3}H-FA)
591 uptake by HTR-8/SVneo cells.

592 Cells were exposed for 24h to metformin (METF 0.01-1 mM; n=7-9) or the respective solvent
593 (control, indicated by the dashed line; n=9) and the effect upon uptake of 10 nM ^{3}H-DG (panel
594 A) or 20 nM ^{3}H-FA (panel B) was evaluated. Shown are arithmetic means±SEM. * P<0.05
595 significantly different from control (one-way analysis of variance followed by Tukey's test).
596

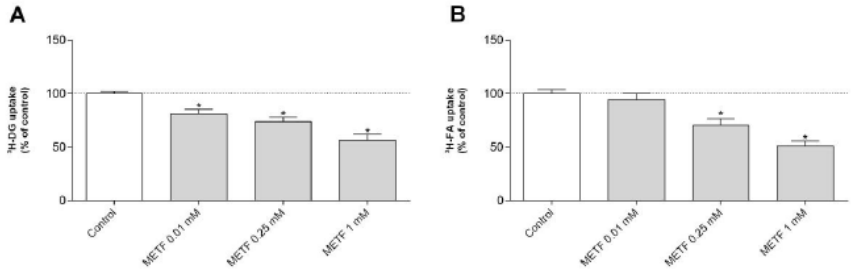
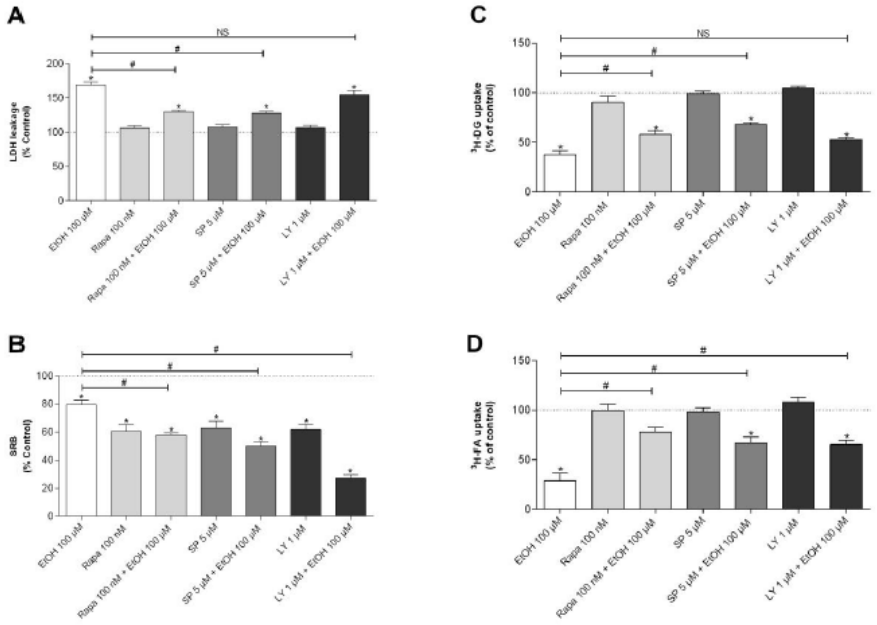


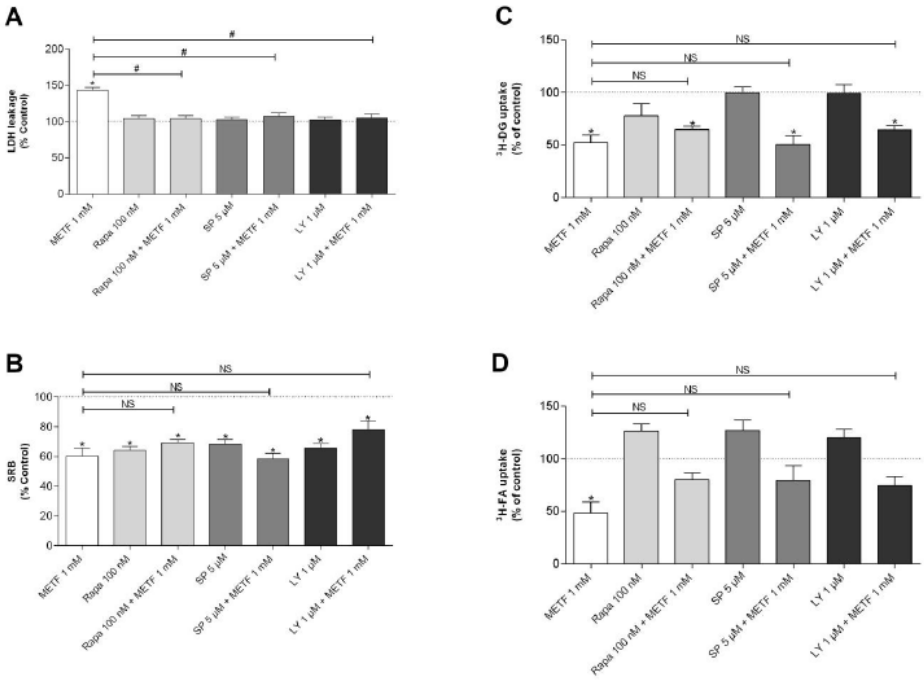
Figure 5 Involvement of mTOR, JNK and PI3K intracellular signaling pathways in the effect of EtOH on HTR-8/SVneo cells.

Cells were exposed for 24h to 100 µM EtOH, 100 nM rapamycin (RAPA), 5 µM SP600125 (SP), 1 µM LY294002 (LY), EtOH+RAPA, EtOH+SP, EtOH+LY, or the respective solvents (control, indicated by the dashed line), and the effect upon cell viability (panel A), cell culture growth (panel B), ^{3}H-DG uptake (panel C) and ^{3}H-FA uptake (panel D), was evaluated. Shown are arithmetic means±SEM (n=9). * P<0.05 significantly different from control (dashed line); $^{\#}$ P<0.05 significantly different from EtOH 100 µM; ^{NS}, not significantly different from EtOH 100 µM (one-way analysis of variance followed by Tukey's test).



607 **Figure 6** Involvement of mTOR, JNK and PI3K intracellular signaling pathways in the
608 effect of METF on HTR-8/SVneo cells.

609 Cells were exposed for 24h to 1 mM METF (n=9), 100 nM rapamycin (RAPA), 5 µM SP600125
610 (SP), 1 µM LY294002 (LY), METF+RAPA, METF+SP and METF+LY, or the respective solvents
611 (control, indicated by the dashed line), and the effect upon cell viability (panel A), cell culture
612 growth (panel B), ^{3}H-DG uptake (panel C) and ^{3}H-FA uptake (panel D) was evaluated. Shown are
613 arithmetic means±SEM (n=8-12). * P<0.05 significantly different from control (dashed line); $^{\#}$
614 P<0.05 significantly different from METF 1 mM; ^{NS}, not significantly different from METF 1 mM
615 (one-way analysis of variance followed by Tukey's test).
616

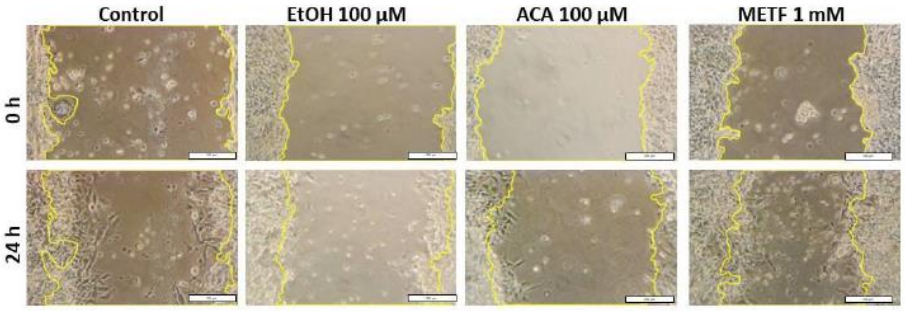


617 **Figure S1** Representative images of cell migration determination by wound healing
618 assay.

619 Cell monolayers were scratched with a pipette tip and then treated for 24 h (in serum-free
620 culture medium) with ethanol (EtOH 100 µM), acetaldehyde (ACA 100 µM), metformin (METF 1
621 mM) or the respective solvents (control). Images shown were obtained at 0h and 24h after
622 injury. Scale bars: 200 µm.

623

Fig. S1



CHAPTER II

Modulation of LC-PUFAs transport and placentation-related processes in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

The information contained in this chapter is included in the following original publication:

Manuscript C. Correia-Branco A, Keating E and Martel F. **Arachidonic Acid Reverses Xanthohumol-Induced Insufficiency in a Human First-Trimester Extravillous Trophoblast Cell Line (HTR-8/SVneo Cells).** Reproductive Sciences. 2017: 1933719117746762.

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Arachidonic Acid Reverses Xanthohumol-Induced Insufficiency in a Human First-Trimester Extravillous Trophoblast Cell Line (HTR-8/SVneo Cells)

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Abstract

We previously described a negative effect of xanthohumol (XN) upon placentation-related processes. We aimed to better characterize this effect by investigating the effect of XN upon the uptake of arachidonic acid (ARA), a crucial nutrient during pregnancy, by the HTR-8/SVneo human first-trimester extravillous trophoblast cell line and its relationship with the negative effect of XN upon placentation-related processes. Uptake of ^{14}C-ARA (100 nM) was time dependent and inhibited by short-term (26 minutes) or long-term (24 hours) exposure to XN. Xanthohumol (24 hours; 5 μM) behaved as an uncompetitive inhibitor of ^{14}C-ARA uptake; the mammalian target of rapamycin, tyrosine kinases, and c-Jun N-terminal kinases intracellular pathways were involved in this effect; and it markedly reduced long-chain acyl-CoA synthetase 1 messenger RNA levels. Moreover, the effects of XN (24 hours; 5 μM) upon cell proliferation, culture growth, migration, viability, and apoptosis index were prevented by high extracellular ARA but not by the peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist rosiglitazone. We thus conclude that ARA is an essential nutrient regulating cell viability, proliferation, culture growth, migration, and apoptosis of HTR-8/SVneo cells and that the deleterious effects of XN involve inhibition of ARA cellular uptake but appears to be independent of PPAR-γ activation.

Keywords

arachidonic acid, placentation, trophoblast cells, xanthohumol, ACSL1

Introduction

Placentation is a continuous and highly regulated process that begins immediately after fertilization and ends only after delivery.[1] An adequate placentation and a consequent satisfactory nutrient supply to the fetus are crucial factors for fetal development and growth and for a good pregnancy outcome. Such critical importance is evident as fetal growth restriction (FGR), and preeclampsia is characterized by impaired uterine blood flow and placental development causing reduced fetal nutrient uptake and fetal hypoxia.[1] Extravillous trophoblasts (EVTs) are the main cell type involved in the placentation process. They are fully specialized trophoblasts exhibiting an invasive and proliferative phenotype, which perform the anchorage of the chorionic villi into the uterine wall and actively regulate uterine spiral arteries remodeling, a process that ends up with the establishment of the uteroplacental blood flow.[2]

The developing placenta and the fetus require the essential fatty acids such as linoleic acid (LA; 18:2n-6) and γ-linolenic acid (γ-LNA; 18:3n-3), which are obtained predominantly from vegetable oils, and their respective long-chain polyunsaturated fatty acids (LC-PUFAs) derivatives, arachidonic acid (ARA; 20:4n-6), and docosahexaenoic acid (DHA; 22:6n-3).[3,4] As reviewed elsewhere,[4] ARA is a major precursor for the synthesis of eicosanoids such as prostaglandins, thromboxanes, and leukotrienes, which are important for the development of fetal nervous, visual, immune, and vascular systems,[4,5] and DHA is crucial for the development of fetal neurovisual system and is highly accumulated in the brain and retina.[4,5] As such, a deficiency of ARA and DHA during intrauterine life is associated with brain and retinal abnormalities in neonates, and recommendation for supplementation during pregnancy with these fatty acids has been suggested.[6]

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Transfer of fatty acids from maternal to fetal circulation may occur by simple diffusion.[7] However, in tissues with high fatty acid demand, uptake of fatty acids by simple diffusion may be insufficient to meet minimum requirements.[7] Although LC-PUFAs cannot synthesized by the fetus, they are present at higher levels in fetal circulation, in relation to maternal circulation. This is consistent with an active and selective placental transfer of these fatty acids in favor of the fetal compartment—a process described as "biomagnification."[7] At the syncytiotrophoblast transporting epithelium level, both passive diffusion and membrane-associated transporters such as placental plasma membrane fatty acid-binding protein, fatty acid transport proteins, and fatty acid translocase are thought to be involved in cellular uptake of LC-PUFAs from the maternal circulation.[5,8] In addition, intracellular long-chain acyl-CoA synthetases (ACSLs), a group of cytosolic enzymes that convert LC-PUFAs into acyl-CoA derivatives for further esterification or β-oxidation, thus preventing the efflux of the incorporated fatty acids, also appear to be involved in LC-PUFAs uptake.[8-10]

Xanthohumol (XN; 3'-[3,3-dimethyl allyl]-2',4',4-trihydroxy-6'-methoxychalcone)—a structurally simple prenylated chalcone that occurs in the hop plant and is used to add bitterness and flavor to beer[11]—has previously been described by us to cause a significant impairment of placentation-related processes by significantly decreasing viability, culture growth, and proliferation of a human first-trimester EVT cell line (HTR-8/SVneo cells).[12] We herein intended to investigate modulation of the uptake of ARA as being involved in the changes in the placentation process induced by XN. We verified that XN inhibits ARA uptake and that ARA is able to revert the negative effects of XH upon placentation.

Materials and Methods

Materials

^{14}C-ARA (arachidonic acid, [1-^{14}C]-ARA; specific activity 55 mCi/mmol), ^{14}C-DHA (docosahexaenoic acid, 4,7,10,13,16,19-[1-^{14}C]-DHA; specific activity 55 mCi/mmol),[14] C-L-MET (^{14}C-L-methionine; specific activity 40-60 mCi/mmol), ^{3}H-FA (folic acid, [3,5,7,9-^{3}H]-FA sodium salt; specific activity 40.0 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, Missouri), ^{3}H-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol; GE Healthcare GmbH, Freiburg, Germany), antibiotic/antimycotic solution (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B), ARA sodium salt, cis-4,7,10,13,16,19-DHA, cis-5,8,11,14,17-eicosapentaenoic acid (EPA), cis-9-octadecenoic acid (oleic acid [OA]), essentially fatty acid-free bovine serum albumin (BSA), genistein, HEPES-NaOH (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), LA, γ-LNA, magnesium sulfate ($MgSO_4$), N-2-hydromorfolin-ethanesulphonic acid (MES), reduced nicotinamide adenine dinucleotide (NADH), palmitic acid sodium salt (PA), p-formaldehyde, potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), rapamycin, Roswell Park Memorial Institute (RPMI) 1640 medium, sodium citrate tribasic dehydrate, sodium chloride (NaCl), rosiglitazone (RG), SP600125, sulforhodamine B (SRB), trichloroacetic acid (TCA), and Triton X-100 and trypsin-EDTA solution (Sigma, St. Louis, Missouri). Fetal bovine serum (Gibco, Life Technologies Corporation, California), D(+)-glucose, dimethyl sulphoxide (DMSO), Tris (tris[hydroxymethyl]-aminomethane hydrochloride), Triton X-100 (Merck, Darmstadt, Germany), triacsin C (Alomone Labs, Jerusalem, Israel), In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany), DNAse I (Amsbio, AMS Biotechnology Limited, United Kingdom), qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, Maryland), KAPA SYBR FAST quantitative polymerase chain reaction (qPCR) Master Mix (KAPA SYBR FAST qPCR Kit Master Mix Universal; Kapa Biosystems, Wilmington, Massachusetts), and NZYol isolation reagent (NZYtech, Lisbon, Portugal). Xanthohumol was kindly donated by Eng. José M. Machado Cruz (Instituto de Bebidas e Saúde [iBeSa], S. Mamede Infesta, Portugal).

The drugs to be tested were dissolved in 1% (vol/vol) acetic acid (sulforhodamine B), DMSO (genistein, rapamycin, RG, SP600125), 100% (vol/vol) ethanol (DHA, EPA, LA, γ-LNA, OA, PA, XN), or 100% (vol/vol) methanol (ARA, triacsin C). The final concentration of these solvents was 1% (vol/vol) in preincubation and incubation buffer and in the culture media. Controls for these compounds were run in the presence of the respective solvents. Stock solutions (ARA 1 M, DHA 1 M, EPA 1 M, genistein 100 mM, LA 100 mM, γ-LNA100 mM, OA 1 M, PA 1 M, rapamycin 1 mM, RG 10 mM, SP600125 10 mM, triacsin C 1 mM, and XN 100 mM) were stored at −20°C, unless otherwise stated.

Human First-Trimester EVT Cell Culture (HTR-8/SVneo Cells)

HTR-8/SVneo cells were generously donated by Dr Charles H. Graham (Department of Anatomy and Cell Biology, Queen's University, Kingston, Canada) and were used between passage number 84 and 112. Cells were maintained in a humidified atmosphere of 5% CO_2—95% air and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution. Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.05% trypsin-EDTA, 5 minutes, 37°C, split 1:6 ratio) and subcultured in plastic culture dishes (21 cm^2, Ø 60 mm; TPP, Trasadingen, Switzerland). For transport experiments and for quantification of cell viability, proliferation, culture growth, and migration, HTR-8/SVneo cells were seeded on 24-well plastic cell culture dishes (2 cm^2, Ø 16 mm; TPP) and were used after 9 to 14 days in culture (90%-100% confluence). For determination of apoptosis index, cells were seeded on glass coverslips on 24-well plastic cell culture dishes (2 cm^2, Ø 16 mm; TPP) and were used after 2 days in culture (20% confluence). For RNA extraction, cells were seeded in plastic culture dishes (21 cm^2, Ø 60 mm; TPP) and

were used after 10 days in culture (95%-100% confluence). In all experiments, the cell medium was made free of fetal calf serum for 24 hours before the experiments. Moreover, in experiments using LC-PUFAs (^{14}C-ARA, ^{14}C-DHA, and ARA), the medium was supplemented with 1% (wt/vol) essentially fatty acid-free BSA for 24 hours before the experiments as previously described by our group.[8]

Transport Studies

Transport experiments were performed in buffer with the following composition (in mM): 12.5 HEPES-NaOH, 12.5 MES, 4.8 KCl, 125 NaCl, 1.2 mM KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 5.6 D(+)-glucose, and pH 7.5 (unless otherwise stated). In ^{3}H-FA uptake experiments, buffer pH was 5.5. In ^{14}C-ARA and ^{14}C-DHA uptake experiments, the buffer was supplemented with 1% (wt/vol) essentially fatty acid-free BSA as previously described by our group.[8]

Initially, the culture medium was aspirated and the cells were washed with buffer at 37°C; then the cell monolayers were preincubated for 20 minutes with buffer at 37°C. Uptake was initiated by the addition of 0.2 mL buffer at 37°C containing 100 nM ^{14}C-ARA (except in experiments for determination of kinetics of ^{14}C-ARA uptake), 500 nM ^{14}C-DHA, 250 nM ^{14}C-L-MET, or 20 nM ^{3}H-FA. In experiments for determination of kinetics of ^{14}C-ARA uptake, cells were incubated for 6 minutes with increasing concentrations (50-1000 nM) of ^{14}C-ARA. Incubation was stopped after 6 minutes (except in the time-course experiments) by removing the incubation medium, placing the cells on ice and rinsing them with 0.5 mL ice-cold buffer without fatty acid-free BSA. The cells were then solubilized with 0.3 mL 0.1% (vol/vol) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed at 4°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Treatment of the Cells

The concentrations of compounds to be tested were chosen based in previous works of our group.[8,12,13] In order to test the short-term effect of compounds, cells were exposed to the compounds during the preincubation and incubation periods (corresponding to a 26-minute exposure time). In order to test the long-term effect of compounds, cells were exposed to the compounds for 24 hours in serum-free culture medium and during the preincubation and incubations periods (corresponding to a 26-minute exposure time).

Protein Determination

The protein content of cell monolayers was determined as described by Bradford,[14] using human serum albumin as standard.

Determination of Cell Viability

The long-term (24 hours) effect of compounds upon the viability of HTR-8/SVneo cells was determined spectrophotometrically by measuring the cellular leakage of the cytosolic enzyme lactate dehydrogenase into the extracellular medium. This was done by quantification of the decrease in the absorbance of NADH during the reduction of pyruvate to lactate as previously described by Bergmeyer and Bernt.[15]

Determination of Cell Proliferation Rates

The long-term (24 hours) effect of compounds upon cellular proliferation rates of HTR-8/SVneo cells was determined with the ^{3}H-thymidine incorporation assay. Briefly, HTR-8/SVneo cells were incubated with ^{3}H-thymidine 0.025 µCi/mL during the last 5 hours of the 24 hours incubation period. After removal of excess ^{3}H-thymidine by a 10% TCA (300 µL) wash for 1 hour at 4°C, drying for 30 minutes, and addition of 280 µL of NaOH 1 M, the incorporated ^{3}H-thymidine was measured by liquid scintillometry.

Determination of Culture Growth

Culture growth was determined by the SRB assay. Briefly, at the end of the long-term treatment (24 hours) period with compounds, 62.5 µL of ice-cold 50% (wt/vol) TCA were added to the culture medium (500 µL) on each well to fix cells (1 hour at 4°C in the dark). The plates were then washed 5 times with tap water to remove TCA. Plates were air dried and then stained for 15 minutes with 0.4% (wt/vol) SRB dissolved in 1% (vol/vol) acetic acid. Sulforhodamine B was removed, and cultures were rinsed 4 times with 1% (vol/vol) acetic acid to remove residual dye. Plates were again air dried, and the bound dye was then solubilized with 375 µL of 10 mM Tris-NaOH solution (pH 10.5). The absorbance of each well was determined at 540 nm.

Determination of Migration Rates

Cell migration rates were determined by the in vitro wound healing assay. Briefly, HTR-8/SVneo cell monolayers were scratched with a 10-µL pipette tip and were afterward treated for 24 hours with compounds to be tested. Images were obtained at 0 and 24 hours of treatment, and quantification was performed by using ImageJ software.[16,17]

Determination of Apoptosis Index (TUNEL Assay)

To determine the apoptosis index, the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed using the In Situ Cell Death Detection kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, after a long-term (24 hours) treatment of the cells with compounds to be tested, the cells were fixed with 4% (wt/vol) p-formaldehyde solution in Phosphate Buffered Saline (PBS) for 30 minutes, permeabilized

with sodium citrate 0.1% (wt/vol) and Triton X-100 0.1% (vol/vol) for 2 minutes at 4°C, and then incubated with fluorescein isothiocyanate-conjugated deoxyuridine triphosphate (dUTP) for 1 hour at 37°C. DAPI (4',6-diamidino-2-phenylindole) (0.5 µg/mL in methanol, 5 minutes) was used to counterstain total nuclei. Coverslips were mounted on glass slides and visualized under a fluorescence microscope (Nikon 50i; Nikon, Japan). The percentage of TUNEL-stained nuclei was evaluated in relation to 700 to 2500 DAPI-stained nuclei observed in 12 to 15 randomly chosen optical fields per slide (in a total of about 4×10^5 nuclei). Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2; United Kingdom). Apoptosis index was calculated as the number of apoptotic cells (in % of control). Coverslips were mounted on glass slides and visualized under a fluorescence microscope (Olympus BH-2, Southend-on-Sea; United Kingdom). The percentage of TUNEL-stained nuclei was evaluated in relation to 700 to 2500 DAPI-stained nuclei observed in 12 to 15 randomly chosen optical fields per slide (in a total of about 4 × 105 nuclei). Apoptosis index was calculated as the number of apoptotic cells (in % of control).

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from HTR-8/SVneo cells treated for 24 hours with compounds to be tested, using NZYol isolation reagent, according to manufacturer's instructions (NZYtech). Before complementary DNA (cDNA) synthesis, total RNA was treated with DNAse I, and 1 µg of the resulting DNA-free RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences) in 20 µL of final reaction volume according to manufacturer's instructions. For quantitative real-time polymerase chain reaction(qRT-PCR), 2 µL of the 20 µL reverse transcription reaction mixture was used. For the calibration curve, HTR-8/SVneo standard cDNA was diluted in 6 different concentrations. The qRT-PCR was carried out using a LightCycler 96 (Roche, Nutley, New Jersey). Ten microliter reactions were set up in 96-well plates using 10 µM of each primer and 5 µL of KAPA SYBR FAST qPCR Master Mix (KAPA SYBR FAST qPCR Kit Master Mix Universal; Kapa Biosystems) according to manufacturer's instructions.

The primer pair used for ACSL1 was 5'-CGA GGG CGA GGT GTG T-3' (forward) and 5'-GTG TAA CCA GCC GTC TTT GTC-3' (reverse). The amount of ACSL1 messenger RNA (mRNA) was normalized to the amount of mRNA of the housekeeping gene, human β-actin. The primer pair used for β-actin was: 5'-AGA GCC TCG CCT TTG CCG AT-3' (forward) and 5'-CCA TCA CGC CCT GGT GCC T-3' (reverse). Cycling conditions for human ACSL1 and β-actin amplification were as follows: denaturation (95°C for 5 minutes), amplification, and quantification (95°C for 10 seconds, annealing temperature [AT; 65°C] for 10 seconds, and 72°C for 10 seconds, with a single fluorescence measurement at the end of the 72°C for 10-second segment) repeated 55 (ACSL1) or 45 times (β-actin), a melting curve program ([AT+10]°C for 15 seconds and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement) and a cooling step to 40°C for 30 seconds. Data were analyzed using LightCycler 96 SW 1.1 analysis software (Roche Diagnostics), and results were analyzed by the ΔΔCt method.[18] β-actin mRNA expression levels were not affected by treatment of the cells (data not shown).

Calculations and Statistics

For the analysis of the time course of uptake, the parameters of Equation (1) were fitted to the experimental data by a nonlinear regression analysis using a computer-assisted method.[19]

$$A(t) = k_{in}/k_{out}(1 - e^{-k_{out} \cdot t}). \quad (1)$$

In Equation (1), $A(t)$ represents the accumulation at time t, k_{in} and k_{out} represent the rate constants for inward and outward transport, respectively, and t represents the incubation time. A_{max} is defined as the accumulation at steady state ($t \rightarrow \infty$).

For the analysis of the saturation curve, the parameters of the Michaelis-Menten equation were fitted to the experimental data by using a nonlinear regression analysis, using a computer-assisted method.[19] For calculation of IC_{50} values, corresponding to the concentration of compound causing 50% of its maximal effect, the parameters of the Hill equation were fitted to the experimental data by using a nonlinear regression analysis, using a computer-assisted method.[19]

Arithmetic means are given with standard error of the mean and geometric means with 95% confidence intervals. n represents the number of replicates of at least 2 different experiments. Statistical significance of the difference between 2 groups was evaluated by the Student t test, and statistical significance of the difference between 3 or more groups was evaluated by 1-way analysis of variance test, followed by Bonferroni test. Differences were considered to be significant when $P < .05$.

Results

Characterization of ^{14}C-ARA Uptake

Time dependence. In a first series of experiments, we determined the time course of ^{14}C-ARA accumulation by HTR-8/SVneo cells. As shown in Figure 1, uptake of ^{14}C-ARA (100 nM) by HTR-8/SVneo cells was time dependent, being linear with time for up to 10 minutes of incubation (Figure 1A inset). Thus, in subsequent experiments, initial rates of ^{14}C-ARA uptake were measured by incubating cells for 6 minutes with ^{14}C-ARA. The time-course parameters calculated are shown in Table 1.

pH dependence. Protonation of long-chain fatty acids facilitates their simple diffusion across the membrane lipid bilayer.[20] As such, we examined the pH dependence of ^{14}C-ARA (100 nM) uptake by HTR-8/SVneo cells, by varying the pH of the preincubation and incubation buffer from 6.5 to 8.5. As shown in

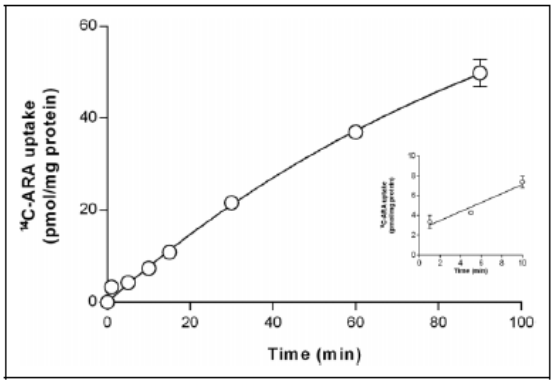

Figure 1. Time course of ^{14}C-ARA uptake by HTR-8/SVneo cells. Cells were preincubated for 20 minutes with buffer at 37°C and then incubated for various periods of time with 100 nM ^{14}C-ARA. Inset highlights linearity of uptake up to the 10th minute of incubation. Shown are arithmetic mean ± SEM (n = 4-8). ARA indicates arachidonic acid; SEM, standard error of the mean.

Table 1. Time-Course Parameters of ^{14}C-ARA, ^{14}C-DHA, ^{14}C-L-Met, and ^{3}H-FA Uptake by HTR-8/SVneo cells.[a]

	A_{max}	k_{in}	k_{out}	n
	(pmol·mg prot^{-1})	(μL mg prot^{-1}·min^{-1})	(min^{-1})	
^{14}C-ARA	89.4 ± 12.4	8.0 ± 0.4	0.0090 ± 0.0017	4-8
^{14}C-DHA	98.4 ± 15.7	5.2 ± 1.0	0.0265 ± 0.009	4-8
^{14}C-L-MET	180.4 ± 6.7	6.9 ± 0.6	0.095 ± 0.010	4
^{3}H-FA	0.62 ± 0.03	1.9 ± 0.2	0.063 ± 0.007	4

Abbreviations: A_{max}, accumulation at the steady-state; ARA, arachidonic acid; DHA, docosahexaenoic acid; FA, folic acid; k_{in}, constant of inward transport; k_{out}, constant of outward transport; MET, methionine; SEM, standard error of the mean.
[a]Values are shown is arithmetic mean ± SEM.

Figure 2A, uptake of ^{14}C-ARA was found to be acidic pH-stimulated, thus pointing to a protonation-stimulated transport of ^{14}C-ARA across the membrane lipid bilayer.

Specificity of the uptake mechanism. In order to study the contribution of ACSL upon ^{14}C-ARA (100 nM) uptake by HTR-8/SVneo cells, we determined the effect of triacsin C, an inhibitor of the activity of ACSL 1, 3, and 4.[9] Triacsin C markedly reduced ^{14}C-ARA uptake (to ~20% of control; Figure 2B).

Next, we determined the effect of several long-chain fatty acids (100 μM) upon ^{14}C-ARA uptake by these cells. As shown in Figure 2C, ^{14}C-ARA uptake was not affected by OA (18:1n-9), DHA (22:6n-3), and EPA (20:5n-3). In contrast, PA (16:0), LA (18:2n-6), and γ-LNA (18:3n-3) decreased ^{14}C-ARA uptake (by 50%-80%) with the following ranking order of potency: LA > γ-LNA > PA.

Altogether, these results show that uptake of ^{14}C-ARA by HTR-8/SVneo cells is: (a) stimulated at an acidic pH, (b) inhibited by saturated fatty acids and by some PUFAs, and (c) markedly dependent on ACSL activity.

The Dietary Polyphenol XN Concentration-Dependently Inhibits ^{14}C-ARA Uptake

We next determined the effect of the dietary polyphenol XN upon ^{14}C-ARA (100 nM) uptake by HTR-8/SVneo cells. As shown in Figure 3A, a short-term exposure (26 minutes) of the cells to XN (1-500 μM) concentration dependently reduced ^{14}C-ARA uptake (IC_{50} = 55.04 [13.2-41.9] μM). Similarly, a longer exposure (24 hours) of the cells to this polyphenol (0.1–10μM) was also able to concentration-dependently reduce ^{14}C-ARA uptake (IC_{50} = 29.05 [0.39-2170] μM; Figure 3B).

Since DHA is another critical LC-PUFA, playing an important role in 1-carbon metabolism, which also involves FA and L-MET,[21] we also investigated the effect of XN upon ^{14}C-DHA, ^{3}H-FA and ^{14}C-L-MET. Uptake of ^{14}C-DHA, ^{3}H-FA, and ^{14}C-L-MET by HTR-8/SVneo cells is time dependent (Supplemental Figure 1A, C, and E). The calculated time-course parameters are shown in Table 1. ^{3}H-FA uptake is markedly acidic pH-stimulated (Supplemental Figure 1B), suggesting that it involves the high-affinity folate: H^+ symporter, proton-coupled folate transporter.[22] As shown in Supplemental Figure 1D and 1F, a short-term exposure (26 minutes) of the cells to XN (1-500 μM) reduced both ^{3}H-FA (20 nM) and ^{14}C-L-MET (250 nM) uptake, respectively. In agreement with a previous observation,[12] these short-term effects of XN do not relate with a decrease in cell viability or proliferation (results not shown). In contrast, a longer exposure (24 hours) of the cells to XN (0.03-5 μM) did not alter ^{3}H-FA (20 nM) and ^{14}C-L-MET (250 nM) uptake (results not shown). Short- and long-term exposure of the cells to XN also affected ^{14}C-DHA (500 nM) uptake by HTR-8/SVneo cells, but this reduction was not concentration dependent (results not shown). As such, we conclude that the effect of XN upon ^{14}C-ARA uptake is specific.

Xanthohumol Is an Uncompetitive Inhibitor of ^{14}C-ARA Uptake

We next evaluated the effect of a long-term exposure (24 hours) of the cells to XN upon the kinetics of ^{14}C-ARA uptake. As shown in Table 2, ^{14}C-ARA uptake was saturable and XN exposure significantly reduced both the V_{max} and the K_m of ^{14}C-ARA uptake, suggesting that it behaves as an uncompetitive inhibitor of ^{14}C-ARA uptake by HTR-8/SVneo cells.

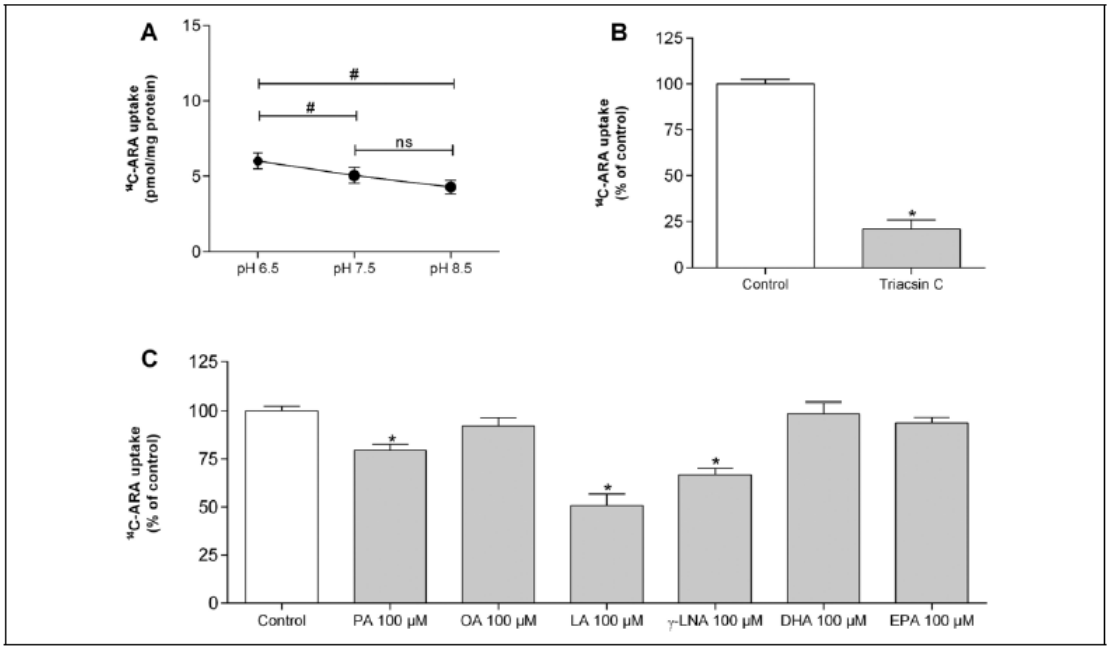

Figure 2. Characterization of ^{14}C-ARA uptake by HTR-8/SVneo cells. Cells were preincubated for 20 minutes with buffer at 37°C and then incubated for 6 minutes with 100 nM ^{14}C-ARA: A, in buffer with a pH ranging from 6.5 to 8.5 (n = 20); B, in the presence of 10 µM triacsin C (n = 20) or the respective solvent (control = 7.45 ± 0.52 pmol/mg protein; n = 20); or C, in the presence of PA (n = 12), OA (n = 12), LA (n = 12), γ-LNA (n = 16), DHA (n = 9), or EPA (n = 17), all at 100 µM or the respective solvent (control = 6.46 ± 0.41 pmol/mg protein; n = 17-20). Shown are arithmetic mean ± SEM. *P < .05 significantly different from control; A, #P < .05 significantly different from each other. ARA indicates arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; ns, not significantly different from each other; OA, oleic acid; PA, palmitic acid; SEM, standard error of the mean.

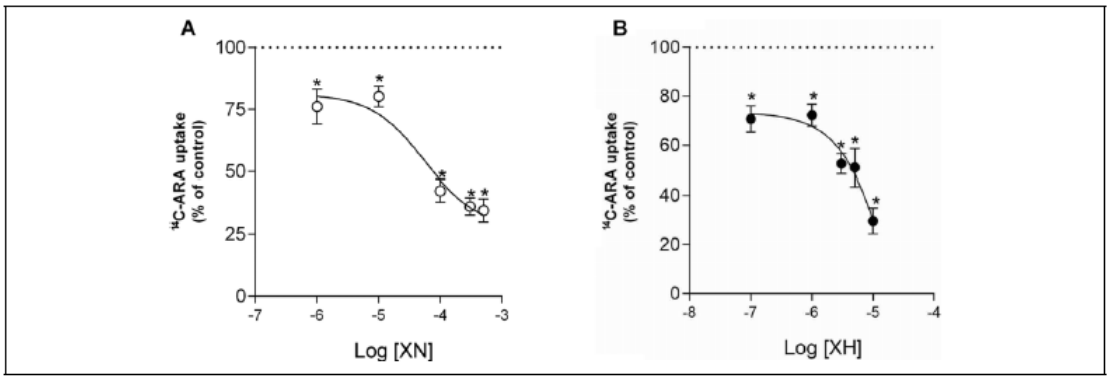

Figure 3. Effect of short-term and long-term exposure to XN upon ^{14}C-ARA uptake by HTR-8/SVneo cells. A, short-term exposure—Cells were preincubated for 20 minutes with buffer at 37°C and then incubated for 6 minutes with 100 nM ^{14}C-ARA in the presence of 1 to 500 µM XN (n = 7-8) or the respective solvent (control = 4.62 ± 0.38 pmol/mg protein, indicated by the dashed line; n = 8). B, long-term exposure—cells were preincubated for 24 hours in serum-free culture medium, preincubated for 20 minutes with buffer at 37°C and then incubated for 6 minutes with 100 nM ^{14}C-ARA in the presence of 0.1 to 10 µM XN (n = 8) or the respective solvent (control = 6.85 ± 0.61 pmol/mg protein, indicated by the dashed line; n = 8). Shown are arithmetic mean ± SEM. *P < .05 significantly different from control (indicated by the dashed line). ARA indicates arachidonic acid; SEM, standard error of the mean; XN, xanthohumol.

Table 2. Kinetic Parameters of ^{14}C-ARA Uptake by HTR-8/SVneo Cells in the Absence (Control) and Presence of a Long-Term Exposure (24 hours) to XN 5 μM.[a]

	V_{max}	K_m	n
	(μmol·mg prot^{-1} 6 min^{-1})	(μM)	
Control	285.1 ± 28.0	2.8 ± 0.4	6
XN (5 μM)	161.8 ± 10.3[b]	1.9 ± 0.2[b]	6

Abbreviations: ARA, arachidonic acid; K_m, Michaelis constant; SEM, standard error of the mean; V_{max}, maximum velocity; XN, xanthohumol.
[a]Values are shown as arithmetic mean ± SEM.
[b]$P < .05$ significantly different from control.

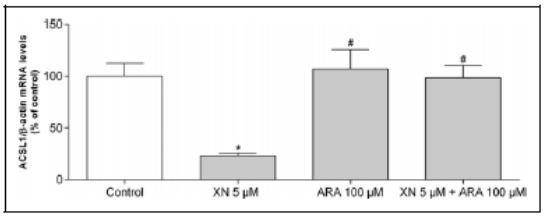

Figure 4. Long-term exposure to XN markedly reduces ACSL1 gene expression, and this effect is reversed by ARA. Cells were exposed for 24 hours (in serum-free culture medium) to 5 μM XN (n = 6-18), 100 μM ARA (n = 6-18) or to a combination of both compounds (XN + ARA; n = 6–18). ARA (100 μM) reversed the effect of 5 μM XN upon ACSL1 mRNA levels. Shown are arithmetic mean ± SEM. *$P < .05$ significantly different from control; #$P < .05$ significantly different from XN 5 μM. ACSL indicates long-chain acyl-CoA synthetase; ARA, arachidonic acid; mRNA, messenger RNA; SEM, standard error of the mean; XN, xanthohumol.

Xanthohumol Downregulates ACSL1 Gene Expression in HTR-8/SVneo Cells and This Effect Is Prevented by ARA

As described above, ^{14}C-ARA uptake is markedly dependent on ACSL activity. So, we decided to investigate the effect of XN upon ACSL1 mRNA expression. As shown in Figure 4, XN (5 μM; 24 hours) markedly downregulated ACSL1 mRNA levels (by ~75%). This inhibitory effect was completely abolished by ARA (100 μM).

Inhibition of ^{14}C-ARA Uptake by XN Involves TK, c-JNK, and mTOR Signaling Pathways

Previous experiments from our group have shown that the effect of XN (5 μM, 24 hours) upon ^{3}H-DG (10 nM) uptake by HTR-8/SVneo cells involved tyrosine kinase (TK), c-Jun N-terminal kinase (JNK), and mammalian target of rapamycin (mTOR) intracellular pathways.[12] In agreement with these previous studies, the effect of XN (5 μM; 24 hours) upon ^{14}C-ARA uptake was diminished in the presence of the TK inhibitor genistein (10 μM) and completely abolished by the JNK inhibitor SP 600125 (5 μM) and by the mTOR inhibitor rapamycin (100 nM), indicating the involvement of TK, JNK, and mTOR intracellular pathways, respectively, in the inhibitory effect of XN upon ^{14}C-ARA uptake (Figure 5). Of note, ^{14}C-ARA uptake by HTR-8/SVneo cells appears to involve TK, JNK, and mTOR intracellular pathways, as inhibitors of these intracellular pathways by themselves diminished ^{14}C-ARA uptake.

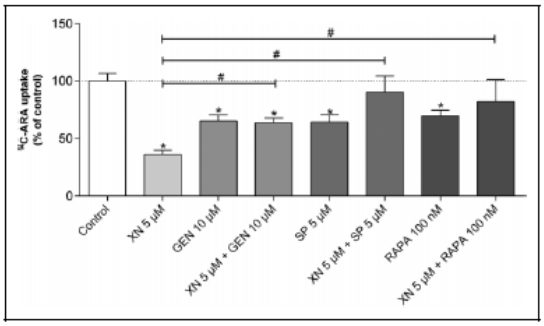

Figure 5. The inhibitory effect of long-term exposure to XN upon ^{14}C-ARA uptake by HTR-8/SVneo cells involves TK, c-JNK, and mTOR signaling pathways. Cells were preincubated for 24 hours in serum-free culture medium, preincubated for 20 minutes with buffer at 37°C, and then incubated with 10 nM ^{14}C-ARA for 6 minutes, in the absence or presence of 5 μM XN (n = 13), 10 μM GEN (n = 13), 5 μM SP(n = 9), 100 nM RAPA (n = 9), XN + GEN (n = 9), XN + SP (n = 9), and XN + RAPA (n = 9) or the respective solvent (control = 13.71 ± 1.42 pmol/mg protein, indicated by the dashed line; n = 16). Shown are arithmetic mean ± SEM. *$P < .05$ significantly different from control, #$P < .05$ significantly different from XN 5 μM. ARA indicates arachidonic acid; GEN, genistein; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; RAPA, rapamycin; SEM, standard error of the mean; SP, SP600125; TK, tyrosine kinase; XN, xanthohumol.

Xanthohumol Inhibits Cell Proliferation, Culture Growth, Cell Migration, Viability, and Increases Apoptosis, and These Effects Are Prevented by ARA

Our group very recently verified that XN (5 μM, 24 hours) markedly reduces HTR-8/SVneo cell proliferation rate, culture growth, viability, and migration capacity.[12] Here, we confirm these results (Figure 6A-D and Supplemental Figure 2A) and further show that XN has a pro-apoptotic effect in these cells (Figure 6E and Supplemental Figure 2B). Interestingly enough, 10 to 100 μM ARA was able to completely abolish the effect of XN in relation to cell proliferation rates (Figure 6A), culture growth (Figure 6B), cell migration (Figure 6C and Supplemental Figure 2A), cell viability (Figure 6D), and apoptosis (Figure 6E and Supplemental Figure 2B), suggesting that ARA cellular availability is protective against XN deleterious effects. Peroxisome proliferator-activated receptor-γ (PPAR-γ) regulates fatty acid transport and storage in human placental trophoblasts[23] and has been described to reduce the invasive,[24] migratory,[25,26] and proliferative capacity of EVTs.[25] We verified that the highly specific PPAR-γ agonist RG (10-100 μM) caused per se an inhibition of cell proliferation, culture growth, migration, viability, and a stimulation of cell apoptosis

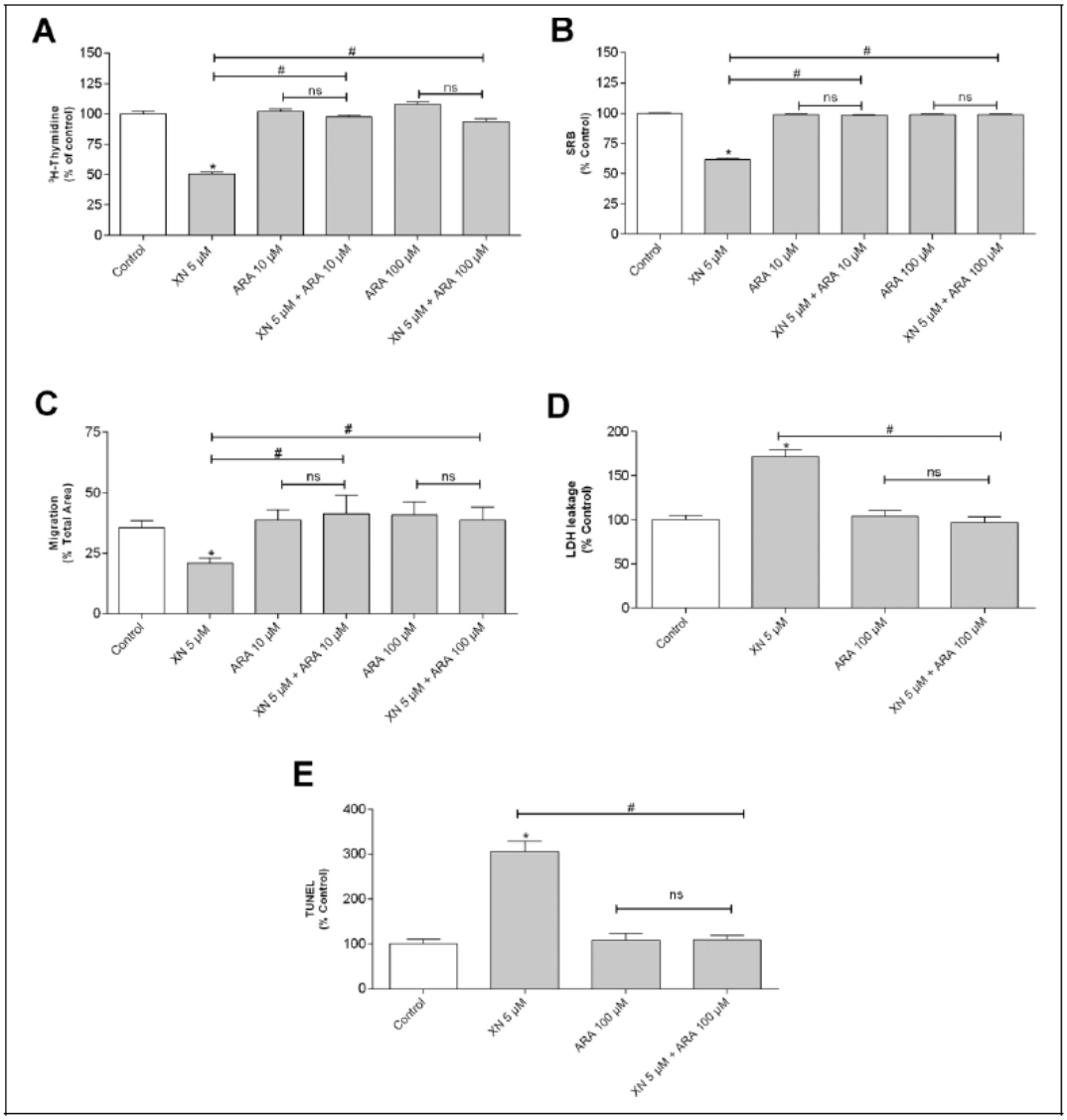

Figure 6. The inhibitory effect of XN upon HTR-8/SVneo cell proliferation, culture growth, migration, viability, and apoptosis is dependent on inhibition of ARA uptake. Cells were exposed for 24 hours (in serum-free culture medium) to 5 µM XN, 10 to 100 µM ARA or to a combination of both compounds (XN + ARA). Arachidonic acid (10 and 100 µM) reversed the effect of 5 µM XN upon cell proliferation (A; control = 0.052 ± 0.002 µCi/mg protein), culture growth (B; abs control = 1.99 ± 0.01), cell migration (C), cell viability (D; control = 20% ± 1% total LDH activity), and cell apoptosis (E; control = 1.68% ± 0.02% apoptotic cells). Shown are arithmetic mean ± SEM (n = 6–18). *P < .05 significantly different from control; #P < .05 significantly different from XN 5 µM. ARA indicates arachidonic acid; LDH, lactate dehydrogenase; ns, not significantly different from each other; SEM, standard error of the mean; XN, xanthohumol.

(Supplemental Figure 3). Moreover, it was not able to reverse the effect of XN in relation to cell proliferation rate (Supplemental Figure 3A), culture growth (Supplemental Figure 3B), migration (Supplemental Figure 3C), viability (Supplemental Figure 3D), apoptosis (Supplemental Figure 3E), and upon ^{14}C-ARA (100 nM; Supplemental Figure 3F) uptake by HTR-8/

SVneo cells. This suggests that these effects of XN are not dependent on PPAR-γ intracellular receptor activation.

Discussion

We recently verified that XN induces an insufficient phenotype in a first-trimester EVT cell line (HTR-8/SVneo cells) in a glucose-dependent manner.[12] An insufficient phenotype characterizes by shallow cellular viability, proliferation, migration, invasion, and angiogenesis and augmented apoptosis index, is associated with an inadequate placentation and is believed to be an underlying mechanism of pregnancy pathologies such as FGR and preeclampsia.[27] In that work, we verified that XN reduces cellular viability, proliferation, culture growth, and cellular migration rates. In the present study, we intended to better characterize this insufficiency-promoting effect of XN, by testing its effect upon the uptake of ARA, another critical nutrient during pregnancy, and by investigating modulation of uptake of ARA as being involved in the negative effect of XN upon placentation, by testing the ability of ARA in preventing/reverting its negative effect upon placentation-related processes, in HTR-8/SVneo cells.

The HTR-8/SVneo cell line has been established by transfecting normal first-trimester trophoblasts with a plasmid containing the gene for the simian virus 40 large T antigen. This cell line keeps the epithelial phenotype and the proliferative and invasive characteristics of first-trimester EVTs, therefore, being an accepted in vitro model system for the study of first-trimester EVTs characteristics.[28]

^{14}C-ARA accumulation by HTR-8/SVneo cells was found to be time dependent. Further characterization of ^{14}C-ARA uptake led us to conclude that a protein-mediated mechanism is involved in this process as demonstrated by the following characteristics: the presence of saturable kinetics, the inhibitory effect of both saturated and unsaturated fatty acids, particularly PUFAs, and the observation that ^{14}C-ARA uptake was strongly (~75%) inhibited by an ACSL1 inhibitor, suggesting that it is dependent on the activity of ACSLs. This last observation is in good agreement with reported data obtained in primary cultured human trophoblasts and in another cellular model of human trophoblasts, the BeWo cell line.[8-10,29] Although simple diffusion is considered quantitatively less important than the above referred protein-mediated transport of fatty acids,[8,30,31] protonation of ^{14}C-ARA and thus simple diffusion of this fatty acid transport across the membrane lipid bilayer also appears to contribute to ^{14}C-ARA uptake by HTR-8/SVneo cells because we observed an increase in ^{14}C-ARA transport with decreasing pH. As to the effect of fatty acids upon ^{14}C-ARA uptake, we observed an inhibitory effect of some fatty acids with the following ranking order of potency: LA > γ-LNA > PA, whereas OA, DHA, and EPA showed no effect. Interestingly enough, these results are in agreement with a study showing that placental plasma membrane–binding sites have a strong preference for LC-PUFAs in the following order: ARA >> LA > γ-LNA >> OA.[32]

Afterward, we tested the effect of XN upon ^{14}C-ARA uptake by HTR-8/SVneo cells. A short-term exposure to this dietary polyphenol potently and concentration dependently inhibited ^{14}C-ARA uptake. The inhibitory effect of XN upon ^{14}C-ARA uptake was very consistent, as long-term (24 hours) exposure of HTR-8/SVneo cells to this chalcone was also able to concentration-dependently reduce ^{14}C-ARA uptake. Xanthohumol was found to inhibit ^{14}C-ARA uptake in an uncompetitive manner (as it reduced both V_{max} and K_m of ^{14}C-ARA uptake). This means that XN binds to the transporter-^{14}C-ARA complex, inhibiting its activity. This causes an increase in the apparent affinity of the transporter for ^{14}C-ARA but results in a decrease in the maximal velocity of ^{14}C-ARA transport. Thus, ^{14}C-ARA binds to the transporter, but there is no effective ^{14}C-ARA uptake into the cells. By using specific inhibitors of intracellular signaling pathways, we concluded that XN inhibits ^{14}C-ARA uptake in a TK-, JNK-, and mTOR-dependent manner. This is very interesting, as we have previously shown that inhibition of uptake of the glucose analog ^{3}H-DG by XN in this same cell line also involves TK, JNK, and mTOR intracellular pathways,[12] and several studies point to the crucial involvement of mTOR and JNK intracellular signaling pathways upon the process of placentation. Indeed, rapamycin, which specifically blocks mTOR, was shown to decrease migration of HTR-8/SVneo cells,[33] and mTOR appears to be essential for the growth and proliferation of early mouse embryos and embryonic stem cells.[34-36] Also, activation of JNK signaling pathway results in changes in the migration and invasion of HTR-8/SVneo cells.[37] Importantly, we verified that ^{14}C-ARA uptake by HTR-8/SVneo cells is dependent on TK, JNK, and mTOR signaling pathways. These data are consonant with emerging evidence, suggesting that mTOR plays a central role as a placental nutrient-sensing signaling mechanism, regulating cell metabolism in response to altered nutrient levels and being influenced by a large number of upstream regulators, such as amino acids, growth factors, and free fatty acids, which are likely to be affected by maternal nutrition.[38-39]

We have previously verified that XN induced glucose deprivation in HTR-8/SVneo cells, which was associated with a significant decrease in cell viability, cell proliferation, culture growth, and cell migratory capacity.[12] To further study the relationship between inhibition of ^{14}C-ARA uptake by XN and the placentation process, we exposed the cells for long term (24 hours) to XN, in the absence and presence of ARA. Interestingly enough, we verified that high extracellular ARA concentrations were able to abolish the effects of XN upon HTR-8/SVneo cellular proliferation, culture growth, migration rates, viability, and cell apoptosis index. Moreover, we observed that XN markedly downregulated ACSL1 gene expression (mRNA levels), and that high extracellular ARA reversed this effect. The involvement of ACSL1 in the inhibitory effect of XN upon ^{14}C-ARA uptake is very interesting, as we verified that ^{14}C-ARA uptake is strongly ACSL1 dependent (as shown by the strong inhibition of ^{14}C-ARA uptake with triacsin C). Interestingly enough, the marked involvement of ACSL1 on ^{14}C-ARA uptake was previously described by our team in term

primary cultured human trophoblasts, and the decrease in ^{14}C-ARA uptake (and also ^{14}C-DHA uptake) observed in trophoblasts obtained from gestational diabetes mellitus pregnancies was also associated with a decrease in ACSL1 mRNA levels.[40] As such, we can conclude that (a) ARA is a crucial nutrient for the placentation process (as evaluated by its effect on placentation-related processes such as cell proliferation, culture growth, migration, viability, and apoptosis) and (b) the inhibitory effect of XN upon the placentation process (as evaluated by its effect on cell proliferation, culture growth, migration, viability, and apoptosis) and upon ACSL1 gene expression is related to a cellular deprivation of ARA, which results from an inhibitory effect of XN upon the cellular uptake of ARA.

Very interestingly, as our group recently verified[12] and as confirmed in this present study, XN induces changes in viability, culture growth, proliferation, migration, and apoptosis of HTR-8/SVneo cells similar to the phenotype observed in insufficient EVTs present in pregnancy-related pathologies such as FGR and preeclampsia. Thus, exposing HTR-8/SVneo cells to XN may constitute an experimental cell model of first-trimester insufficient EVTs, which is still lacking. Of note, the concentration of XN shown here to induce an insufficient phenotype (5 µM) is well above the blood levels found in humans, even after drinking beer, which is the major dietary source of XN.[41]

The LC-PUFAs have been shown to exert their cellular effects through interaction with transcription factors such as PPARs.[42-44] The PPARs are crucial nuclear receptors that include the isoforms PPAR-α, PPAR-β/-δ, and PPAR-γ, all of which bind to DNA as heterodimers with retinoid X receptors, regulating the transcription of a variety of genes involved in lipid metabolism and cellular differentiation.[44,45] The PPAR-γ has been found to regulate uptake of fatty acids, formation of lipid droplets, and de novo lipid synthesis in rat trophoblasts and placental tissues.[23,24,46,47] Moreover, PPAR-γ was shown to play a major role in trophoblast differentiation and early placental development,[47,48] and to inhibit invasion,[24,49] migration,[25,26] and proliferation[25] of EVTs. However, PPAR-γ activation by the highly specific PPAR-γ agonist RG was described to improve preeclampsia in rat models.[50,51] So, we decided to investigate whether the effect of XN on the placentation process was mediated by PPAR-γ. We verified that RG did not interfere with the inhibitory effect of XN upon ^{14}C-ARA uptake, cell proliferation, culture growth, migration, viability, and apoptosis. So, we conclude that the effect of XN on placentation-related processes is not dependent on PPAR-γ activation. Worth of note is the fact that RG showed an inhibitory effect upon these parameters. Thus, it appears that, in agreement with the works described above, there is an inhibitory effect of PPAR-γ activation upon the placentation process.

In conclusion, XN seems to be a potent inhibitor of ARA uptake by HTR-8/SVneo cells. This inhibitory effect is of uncompetitive nature, involves a strong downregulation of ACSL1 mRNA levels, and involves the mTOR, TK, and JNK intracellular signaling pathways. Moreover, the decrease in cell proliferation, culture growth, cell viability, and cell migration; the increase in apoptosis index; and the decrease in ACSL1 mRNA levels induced by XN were abolished by high ARA extracellular concentrations. These results strongly support the conclusion that ARA cellular availability is protective against XN deleterious effects, thus pointing to the critical involvement of ARA upon the placentation process. So, we demonstrate for the first time that ARA is of major importance for the process of placentation, as evidenced by its ability to block the negative impact of XN upon cellular proliferation, viability, culture growth, cellular migration rates, and cellular apoptosis. Interestingly enough, we very recently verified that the inhibitory effect of XN upon HTR-8/SVneo placentation-related processes (cell proliferation, culture growth, cell viability, and migration) was reversed by high extracellular glucose concentration.[12] So, the inhibitory effect of XN upon the placentation process appears to be dependent on a reduction in the cellular uptake of distinct essential nutrients.

Authors' Note

A Correia-Branco contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and drafting of the article. E. Keating contributed to conception and design of the study, data analyses and discussion, and revised the article for important intellectual content. F. Martel contributed to conception and design of the study, data analyses and discussion, revised the article for important intellectual content, and gave the final approval of the version to be published.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplementary material for this article is available online.

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Fig. S1

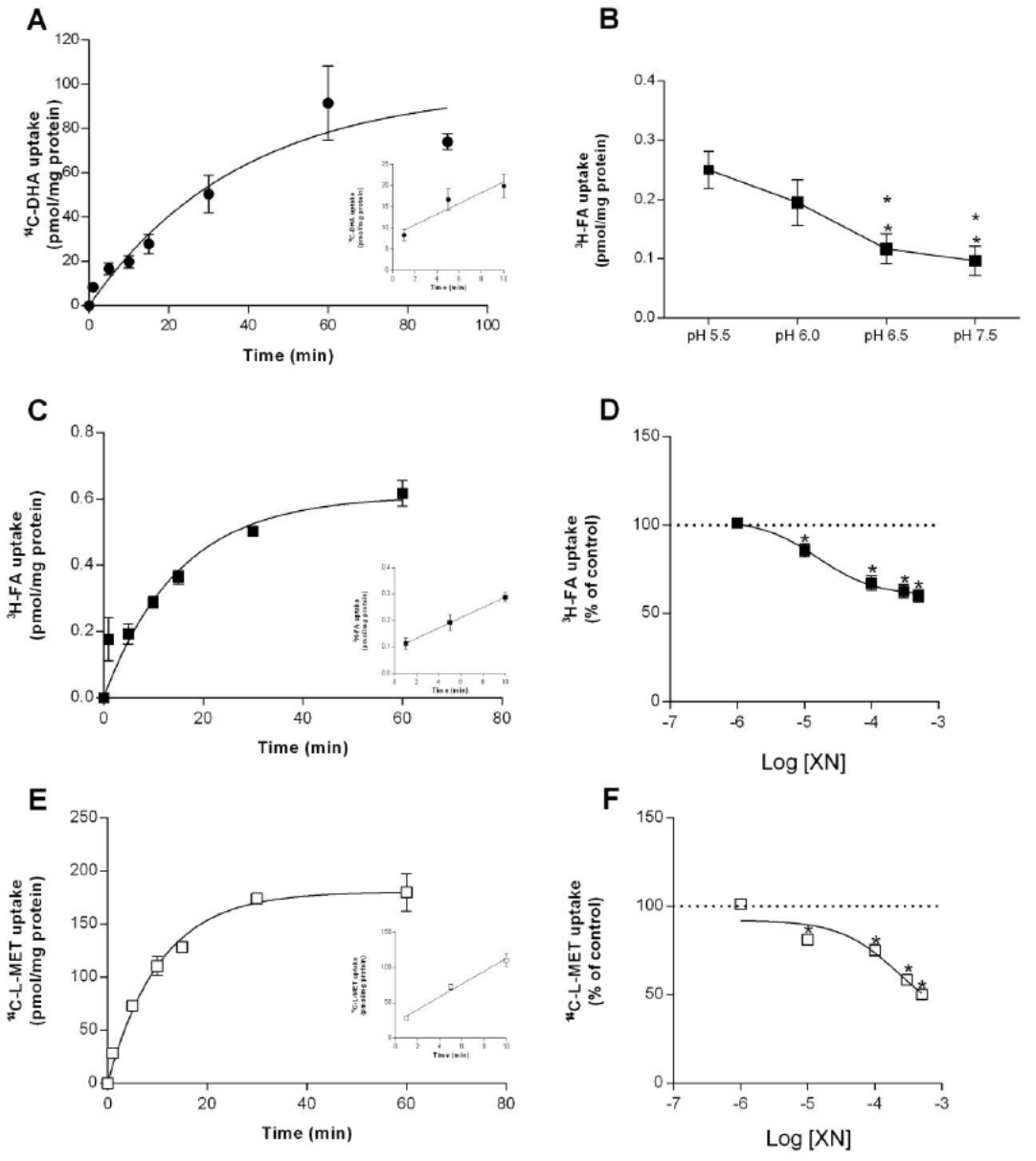
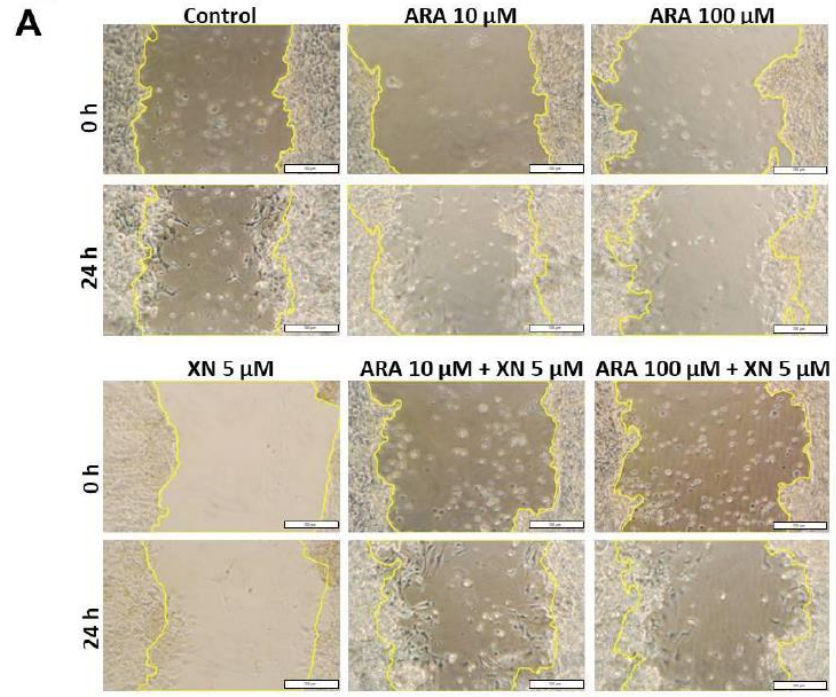

A
^{14}C-DHA uptake (pmol/mg protein)
Time (min)
B
^{3}H-FA uptake (pmol/mg protein)
pH 5.5
pH 6.0
pH 6.5
pH 7.5
C
^{3}H-FA uptake (pmol/mg protein)
Time (min)
D
^{3}H-FA uptake (% of control)
Log [XN]
E
^{14}C-L-MET uptake (pmol/mg protein)
Time (min)
F
^{14}C-L-MET uptake (% of control)
Log [XN]

Figure S1 Characterization of ^{14}C-DHA, ^{3}H-FA and ^{14}C-L-MET and uptake by HTR-8/SVneo cells and effect of short- and long-term exposure to XN.

(A) Time-course of ^{3}H-FA uptake by HTR-8/SVneo cells. Cells were preincubated for 20 min with buffer at 37°C and then incubated for various periods of time with 500 nM ^{14}C-DHA. Inset highlights linearity of uptake up to the 10th min of incubation. Shown are arithmetic means±SEM (*n*=4-8). (B) pH-dependence of ^{3}H-FA uptake by HTR-8/SVneo cells. The extracellular pH in the preincubation and incubation media ranged from 5.5 to 7.5 (*n*=6). (C) Time-course of ^{3}H-FA uptake by HTR-8/SVneo cells. Cells were preincubated for 20 min with buffer at 37°C and then incubated for various periods of time with 20 nM ^{3}H-FA (*n*=4). Inset highlights linearity of uptake up to the 10th min of incubation. (D) Short-term effect of XN upon ^{3}H-FA uptake by HTR-8/SVneo cells. Cells were preincubated for 20 min with buffer at 37°C and then incubated for 6 min with 20 nM ^{3}H-FA, in the presence of 1-500 µM XN (*n*=4-10) or the respective solvent (control=0.28±0.00 pmol/mg prot, indicated by the dashed line; *n*=10). (E) Time-course of ^{14}C-L-MET uptake by HTR-8/SVneo cells. Cells were preincubated for 20 min with buffer at 37°C and then incubated for various periods of time with 250 nM ^{14}C-L-MET (*n*=4). Inset highlights linearity of uptake up to the 10th min of incubation. (F) Short-term effect of XN upon ^{14}C-L-MET uptake by HTR-8/SVneo cells. Cells were preincubated for 20 min with buffer at 37°C and then incubated for 6 min with 250 nM ^{14}C-L-MET, in the presence of 1-500 µM XN (*n*=8-11) or the respective solvent (control=82.51±3.41 pmol/mg prot, indicated by the dashed line; *n*=10). Shown are arithmetic means±SEM. * *P*<0.05 significantly different from control (dashed line); (B) * *P*<0.05 significantly different from pH 5.5.

Fig. S2


A
Control
ARA 10 μM
ARA 100 μM
0 h
24 h
XN 5 μM
ARA 10 μM + XN 5 μM
ARA 100 μM + XN 5 μM
0 h
24 h

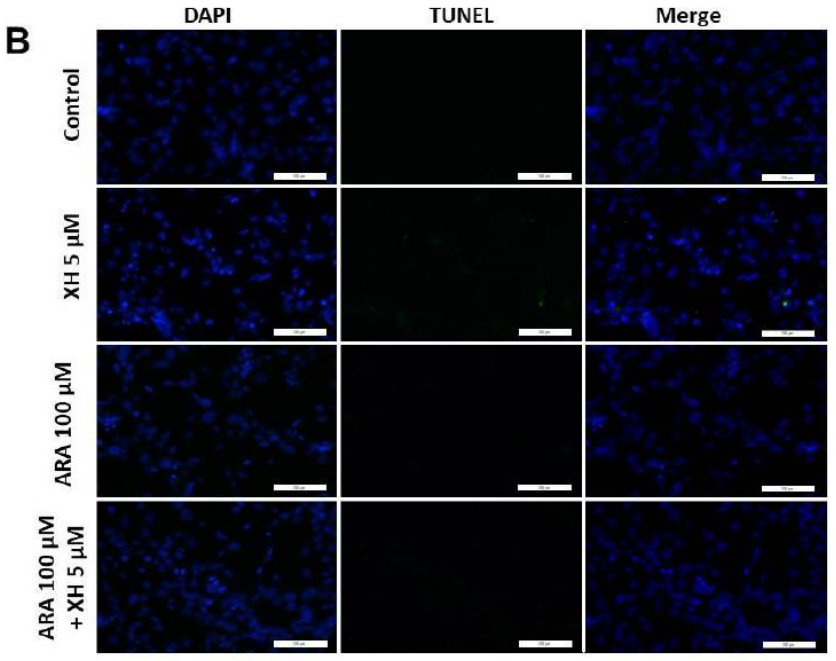

B
DAPI
TUNEL
Merge
Control
XH 5 μM
ARA 100 μM
ARA 100 μM + XH 5 μM

Figure S2 The inhibitory effect of XN upon HTR-8/SVneo cell migration and apoptosis is dependent on inhibition of ARA uptake

Cells were exposed for 24h (in serum-free culture medium) to 5 µM XN, 10-100 µM arachidonic acid (ARA) or to a combination of both compounds (XN + ARA). (A) Representative images of cell migration determination by injury assay. Images shown were obtained at 0h and 24 h after injury. Scale bars: 200 µm. (B) Representative image of cell apoptosis determination by TUNEL assay. Scale bars: 200 µm.

Fig. S3

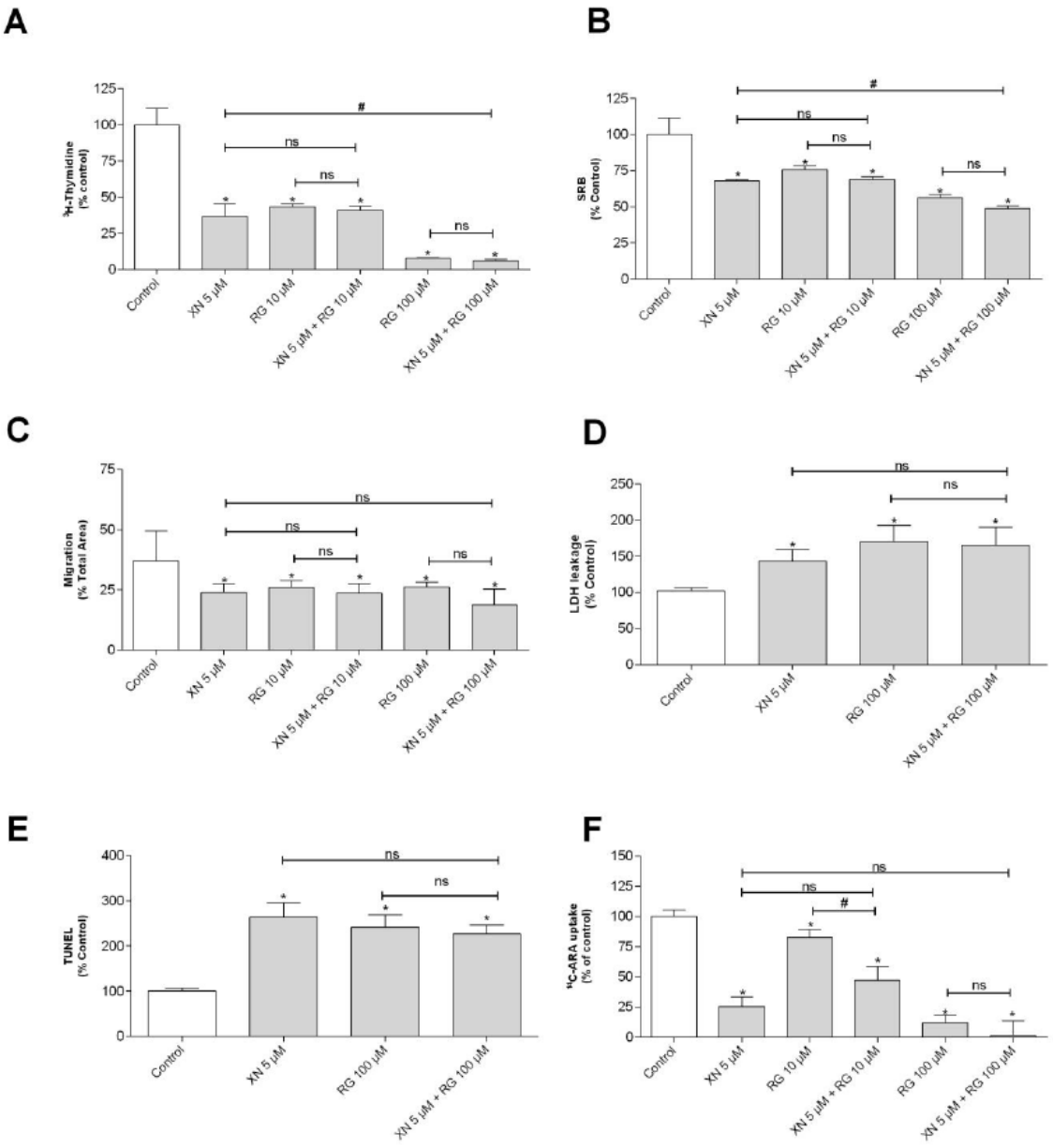

A
B
C
D
E
F
³H-Thymidine (% control)
SRB (% Control)
Migration (% Total Area)
LDH leakage (% Control)
TUNEL (% Control)
¹⁴C-ARA uptake (% of control)
Control
XN 5 µM
RG 10 µM
XN 5 µM + RG 10 µM
RG 100 µM
XN 5 µM + RG 100 µM
ns
#

Figure S3 The inhibitory effect of XN upon HTR-8/SVneo cell proliferation, culture growth, migration, viability, apoptosis and ^{14}C-ARA uptake is not dependent on PPAR-γ intracellular receptors.

Cells were exposed for 24h (in serum-free culture medium) to 5 µM XN (*n*=6-18), 10-100 µM rosiglitazone (RG; *n*=6-18), or to a combination of both compounds (XN + RG; *n*=6-18). RG (10 and 100 µM) did not reverse the effect of 5 µM XN upon cell proliferation (panel A), culture growth (panel B), cell migration (panel C), cell viability (panel D) and cell apoptosis (panel E). F: Cells were exposed for 24h (in serum-free culture medium), preincubated for 20 min with buffer at 37°C and then incubated for 6 min with 100 nM ^{14}C-ARA, in the absence (control=2.34±0.14 pmol/mg prot; *n*=16) or presence of 5 µM XN (*n*=16), 10-100 µM rosiglitazone (RG; *n*=12-16) or a combination of both compounds (XN + RG; n=12-16)). Shown are arithmetic means±SEM. * $P<0.05$ significantly different from control; $^{\#}$ $P<0.05$ significantly different from each other; ns, not significantly different from each other.

CHAPTER III

Modulation of FA transport in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

The information contained in this chapter is included in the following original publication:

Manuscript B. Correia-Branco A, Keating E and Martel F. **Involvement of mTOR, JNK and PI3K in the negative effect of ethanol and metformin on the human first-trimester extravillous trophoblast HTR-8/SVneo cell line**. European Journal of Pharmacology, 2018 (accepted).

European Journal of Pharmacology (Eur J Pharmacol)
DOI: 10.1093/molehr/gav043
IF: 2.896

Manuscript C. Correia-Branco A, Keating E and Martel F. **Arachidonic Acid Reverses Xanthohumol-Induced Insufficiency in a Human First-Trimester Extravillous Trophoblast Cell Line (HTR-8/SVneo Cells).** Reproductive Sciences. 2017: 1933719117746762.

Reproductive Sciences (Reprod Sci)
DOI: 10.1177/1933719117746762
IF: 2.443

CHAPTER IV

Modulation of L-MET transport in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

The information contained in this chapter is included in the following original publication:

Manuscript C. Correia-Branco A, Keating E and Martel F. **Arachidonic Acid Reverses Xanthohumol-Induced Insufficiency in a Human First-Trimester Extravillous Trophoblast Cell Line (HTR-8/SVneo Cells).** Reproductive Sciences. 2017: 1933719117746762.

Reproductive Sciences (Reprod Sci)
DOI: 10.1177/1933719117746762
IF: 2.443

CHAPTER V

Association of modulation by xenobiotics of placentation-related processes with altered placental transport of nutrients, in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

The information contained in this chapter is included in the following original publications:

Manuscript D. Correia-Branco A, Keating E and Martel F. **Placentation-related processes in a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) are affected by several xenobiotics.** Drug and Chemical Toxicology, 2018, 1-5.

Drug and Chemical Toxicology (Drug Chem Toxicol)
DOI: 10.1080/01480545.2018.1463240
IF:1.732

Manuscript D. Correia-Branco A, Keating E and Martel F. **Placentation-related processes in a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) are affected by several xenobiotics.** Drug and Chemical Toxicology, 2018, 1-5.

DRUG AND CHEMICAL TOXICOLOGY, 2018
https://doi.org/10.1080/01480545.2018.1463240

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RESEARCH ARTICLE

Placentation-related processes in a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) are affected by several xenobiotics

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ABSTRACT

Our aim was to investigate the effect of some xenobiotics on placentation-related processes in an extravillous trophoblastic cell line (HTR-8/SVneo cells). Amphetamine, MDMA, theophylline, and fluoxetine, but not nicotine, cocaine, and caffeine, had a negative effect on cell proliferation rates, culture growth, viability, or migratory capacity. These compounds have a detrimental effect in placentation-related processes of HTR-8/SVneo cells.

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Extravillous trophoblasts; HTR-8/SVneo cell line; proliferation; viability; migration; xenobiotics

Introduction

Pregnant women are frequently exposed to several xenobiotics due to lifestyle factors such as diet, smoking, drug abuse, and therapeutic drug use. It is widely accepted that the consumption of drugs of abuse exerts deleterious effects on the fetus (Ganapathy 2011). Moreover, pregnant women often take medication for the treatment of various conditions such as epilepsy, hypertension, depression, and type II and gestational diabetes. However, the chronic effects of these xenobiotics upon the placentation process are still largely unexplored.

In the present work, we aimed to investigate the influence of some drugs of abuse, dietary compounds, and therapeutic drugs on placentation-related processes (cell viability and proliferation, culture growth, migratory capacity, and apoptosis index) of a human first-trimester extravillous trophoblasts (EVTs) cell line (HTR-8/SVneo cells). EVTs are fully specialized trophoblasts displaying an invasive and proliferative phenotype, and they are the key cell type involved in the placentation process (Ji *et al.* 2013). The HTR-8/SVneo cell line displays an unlimited life-span in culture and retains characteristics of invasive trophoblasts such as expression of cytokeratin 18 and some EVT-specific integrins (Graham *et al.* 1993, Knofler 2010). A recent study showed that HTR-8/SVneo cell line contains two mixed populations of cells, namely trophoblast and stromal/mesenchymal cells (Abou-Kheir *et al.* 2017). Nevertheless, this cell line is well established as a suitable *in vitro* model system of first-trimester EVTs.

The following substances were tested: (a) the drugs of abuse amphetamine (AMPH), ecstasy (3,4-methylenedioxymethamphetamine; MDMA), nicotine (NICO), and cocaine (COCA); (b) the methylxanthines caffeine (CAF) and theophylline (TEO), present in drinks such as coffee and tea; and (c) the antidepressant drug fluoxetine (FLUOX). These compounds were chosen because their consumption/exposure during pregnancy is relatively frequent, and because our group previously verified that these compounds affect nutrient uptake by term-trophoblasts (Araújo *et al.* 2008, Keating *et al.* 2008, 2009, Correia-Branco *et al.* 2015). So, we found interesting to evaluate their effect on placentation-related processes.

Materials and methods

Materials

^{3}H-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH, Freiburg, Germany); amphetamine (AMPH), antibiotic/antimicotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B), caffeine (CAF), cocaine (COCA), fluoxetine hydrochloride (FLUOX), HEPES-NaOH (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), MDMA (3,4-methylenedioxy-N-methamphetamine), magnesium sulfate ($MgSO_4$), MES (N-2-hydromorfolinethanesulfonic acid), NADH (reduced nicotinamide adenine dinucleotide), (-)-nicotine hydrogen tartrate salt (NICO), p-formaldehyde, potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), RPMI 1640 medium, sodium citrate tribasic dehydrate, sodium chloride (NaCl), sulforhodamine B (SRB), theophylline (TEO), trichloroacetic acid (TCA), triton X-100, and trypsin-EDTA solution (Sigma, St. Louis, MO). Fetal bovine serum (Gibco, Life Technologies Corporation, Carlsbad,

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CA), D(+)-glucose (DG), DMSO (dimethylsulfoxide), Tris (tris-(hydroxymethyl)-aminomethane hydrochloride), Triton X-100 (Merck, Dramstad, Germany).

Solutions for the drugs to be tested were prepared in water (COCA, FLUOX, NADH, NICO), DMSO (CAF, TEO), or 100% (v/v) methanol (AMPH, MDMA) and were added to the culture medium in a 1% (v/v) final concentration. Controls for the drugs contained the respective solvents. Stock solutions (AMPH 7.4 mM, COCA 100 mM, MDMA 5.2 mM,) were stored at −20 °C, unless otherwise stated. AMPH, MDMA, NICO, COCA, CAF, TEO, and FLUOX were tested in concentrations previously essayed by our group (Correia-Branco *et al.* 2015).

Human first-trimester EVT cell culture (HTR-8/SVneo cells)

HTR-8/SVneo cells were generously donated by Dr. Charles H. Graham (Department of Anatomy & Cell Biology, Queen's University, Kingston, Canada) and were used between passage number 77 and 92. Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution, as previously described by our group (Correia-Branco *et al.* 2015, 2017). The effect of drugs on cell viability, proliferation, culture growth and migration was investigated in HTR-8/SVneo cells cultivated in 24-well plastic cell culture dishes (2 cm^2; Ø 16 mm; TPP®) for 9–14 days (90–100% confluence). The effect of drugs on apoptosis index was investigated in cells seeded on glass coverslips placed on 24-well plastic cell culture dishes (2 cm^2; Ø 16 mm; TPP®) for 2 days (20% confluence). Fetal calf serum was removed from the culture medium in the last 24 h of culture.

Determination of cell viability

The long-term (24h) effect of compounds upon cellular viability was determined by quantification of extracellular activity of LDH, as previously described (Bergmeyer and Bernt 1974, Correia-Branco *et al.* 2015, 2017).

Determination of cell proliferation rates

The long-term (24h) effect of compounds upon cellular proliferation rates was determined with the ^{3}H-thymidine incorporation assay, as previously described (Correia-Branco *et al.* 2015, 2017).

Determination of culture growth

The long-term (24h) effect of compounds on culture growth was determined by the SRB assay, as described elsewhere (Correia-Branco *et al.* 2015, 2017).

Determination of migration rates

The long-term (24h) effect of compounds on cell migration rates was determined by the *in vitro* wound healing assay, as previously described (Correia-Branco *et al.* 2015, 2017). Quantification of images was performed as described (Reinhart-King 2008, Negrao *et al.* 2013).

Determination of apoptosis index (TUNEL assay)

The long-term (24h) effect of compounds on the apoptosis index (number of apoptotic cells [in % of control]) was determined by the TUNEL (terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick-end labeling) assay, as described elsewhere (Araújo *et al.* 2013, Correia-Branco *et al.* 2017).

Protein determination

Determined as described by Bradford (1976), using human serum albumin as standard.

Calculations and statistics

Arithmetic means are given with SEM. *n* represents the number of replicates of at least two different experiments. Statistical significance was evaluated with the Student's *t*-test, and $p < 0.05$ was considered significant.

Results and discussion

Drugs of abuse are an important class of xenobiotics in the context of this study. Indeed, the placenta and the fetus become frequently exposed to drugs of abuse because of maternal use of these drugs. For instance, in an US study, nearly 25% of pregnant women had a positive toxicology screen for any illicit drug and nearly 10% had a positive screen for cocaine, amphetamines, and/or opiates (Schuchat 2017). Importantly enough, these drugs freely cross the placenta barrier, being associated with increased maternal and (or) fetal morbidity and mortality, higher risks of low birth weight, preterm delivery, and teratogenesis (Wright and Walker 2001, Neri *et al.* 2015). We tested AMPH, MDMA, and COCA in concentrations higher than the usually observed in the plasma of chronic consumers, which are 0.03 μM, 0.1 μM, and 0.033 μM, respectively (Verstraete 2004). We verified that AMPH decreased cell proliferation rates in a concentration-dependent manner, while MDMA caused a concentration-independent decrease in this parameter (Figure 1(A)). In contrast, COCA concentration-dependently increased cell proliferation rates, and NICO had no significant effect (Figure 1(A)). MDMA (10 μM) reduced cell viability (Figure 1(B)), culture growth (Figure 1(C)), and cell migration (Figure 1(D)); AMPH (10 μM) also reduced cell viability (Figure 1(B)) and culture growth (Figure 1(C)), but no effect on cell migration was found (Figure 1(D)). In contrast, COCA (25 μM) increased cell viability (Figure 1(B)), culture growth (Figure 1(C)), and apoptosis index (Figure 1(E)), without affecting cell migration (Figure 1(D)). The results obtained with COCA, i.e., increased cell apoptosis associated with an increase in cell proliferation and viability, may strike as paradoxical. However, in our study, cell viability was quantified by the LDH assay, which quantifies cell necrosis. Cell apoptosis and necrosis are separate

modes of cell death with distinct morphological characteristics and that can be triggered independently (Orrenius *et al.* 2011). Depending on the type of cellular injury and on its severity (i.e., concentration of the toxic agent), cell death in the form of either apoptosis or necrosis can be triggered. Most often, exposure to low doses results in apoptosis, whereas higher levels causes necrosis (Kerr 1995, Orrenius *et al.* 2011). In our study, the COCA concentration used stimulated cell proliferation, did not cause cellular necrosis, and increased the amount of apoptotic cells. The increase in apoptosis associated with a proproliferative effect might help to eliminate cells that were produced in excess, that have developed improperly, or that have sustained genetic damage. Alternatively, the increase in apoptosis might be independent from the proproliferative effect, and related to the fact that COCA affects Ca^{2+} homeostasis (Brailoiu *et al.* 2015), which can trigger the apoptotic response (Orrenius *et al.* 2011). Finally, NICO (10 μM) showed no significant effect upon any of the other parameters studied, even using supra-physiological concentrations of this drug of abuse (plasma levels range between 0.05 and 0.19 μM) (Russell *et al.* 1975). Overall, these results show that MDMA and AMPH present deleterious effects on several placentation-related parameters, and reinforce the harmfulness of these drugs of abuse to placental function and fetal health.

Another class of xenobiotics tested were the methylxanthines. Drinks containing CAF and TEO (e.g., coffee and black tea) are often consumed by pregnant women; in the USA, 75% of pregnant women consume low to moderate amounts of CAF (Bracken *et al.* 2003). Methylxanthines readily pass the placenta barrier (Grosso and Bracken 2005) and thus might affect the fetus; thus, their effect on the placenta and fetus

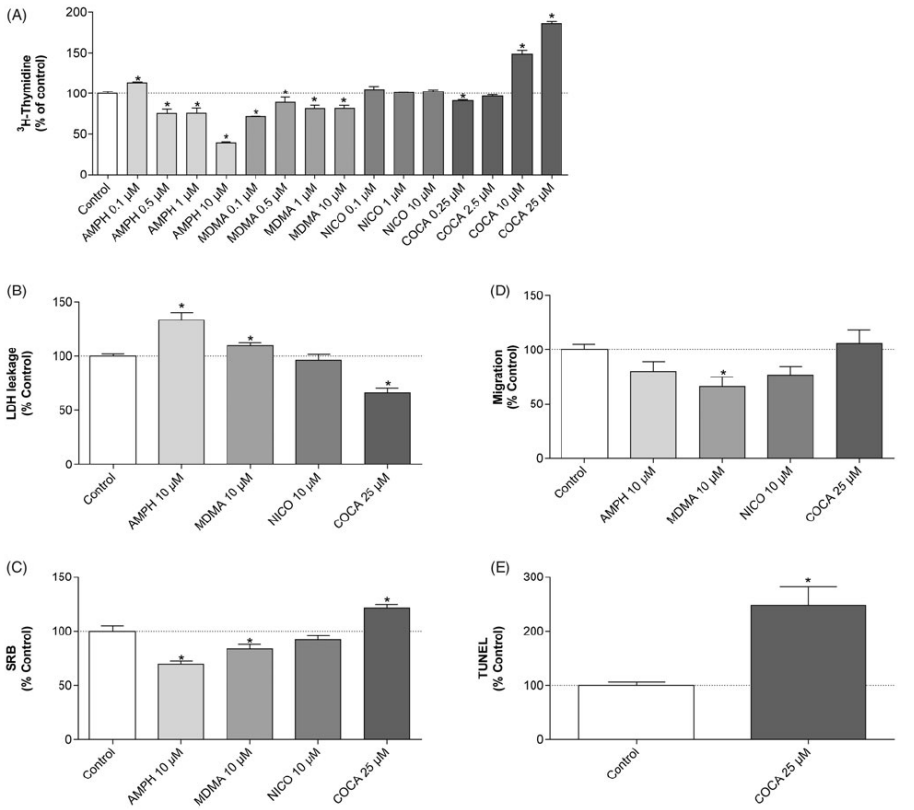

Figure 1. Characterization of the effect of several drugs of abuse upon cell proliferation, cell viability, cell culture growth, and cell migration of HTR-8/SVneo cells.
(A) Cells were exposed for 24 h to increasing concentrations of amphetamine (AMPH 0.1–10 μM; $n = 4$–8), ecstasy (MDMA 0.1–10 μM; $n = 4$–9), nicotine (NICO 0.1–10 μM; $n = 4$), and cocaine (COCA 0.25–25 μM; $n = 4$–8) or the respective solvents (control, indicated by the dashed line; $n = 11$–35). (B–D) Cells were exposed for 24 h to amphetamine (AMPH 10 μM; $n = 8$–15), ecstasy (MDMA 10 μM; $n = 8$–12), nicotine (NICO 10 μM; $n = 8$–12), cocaine (COCA 25 μM; $n = 6$–14) or the respective solvents (control, indicated by the dashed line; $n = 6$–17), and the effect upon cell viability (B), cell culture growth (C), and cell migration (D) was evaluated. (E) Cells were exposed for 24 h to cocaine (COCA 25 μM; $n = 18$) or the respective solvents (control, indicated by the dashed line; $n = 22$) and the effect upon cell apoptosis index was evaluated. Shown are arithmetic means ± SEM. *$p < 0.05$ significantly different from control (Student's *t*-test).

should be explored. The mean plasma levels of CAF and TEO range between 5 and 83 μM (Moffat *et al.* 2004, Youngberg et al. 2011) and 56 and 111 μM, respectively (Moffat *et al.* 2004, Barnes 2013). A 24h-exposure to CAF increased the cell proliferation rates (Figure 2(A)), and CAF (300 μM) also increased the % of viable cells (Figure 2(B)) and culture growth (Figure 2(C)); however, it had no effect upon cell migration (Figure 2(D)). These results clearly show that CAF presents a proproliferative and cytoprotective effect. However, these results must be considered with caution, as there is still some controversy concerning the risks associated with the maternal use of CAF during pregnancy (e.g., spontaneous abortion and fetal growth restriction), and the FDA recommends that pregnant women should avoid the ingestion of CAF (Bracken *et al.* 2003, Jarosz *et al.* 2012). In contrast, TEO (300 μM) did not alter proliferation rates (Figure 2(A)), but reduced cell viability (Figure 2(B)), culture growth (Figure 2(C)), and migration (Figure 2(D)). So, CAF appears to have a placentation-promoting effect while TEO has a cytotoxic and antimigratory effect. Because the concentrations used for both CAF and TEO (300 μM) were higher than the observed on plasma, these results must be analyzed with caution, as the plasma concentrations might not lead to such effects.

Finally, we also tested the effect of the therapeutic drug FLUOX. This compound presented an antiproliferative (Figure 2(A)) and cytotoxic (Figure 2(B)) effect in HTR-8/SVneo cells, resulting in a decrease in culture growth (Figure 2(C)) although it was devoid of effect on cell migration (Figure 2(D)). FLUOX is an antidepressant drug belonging to the

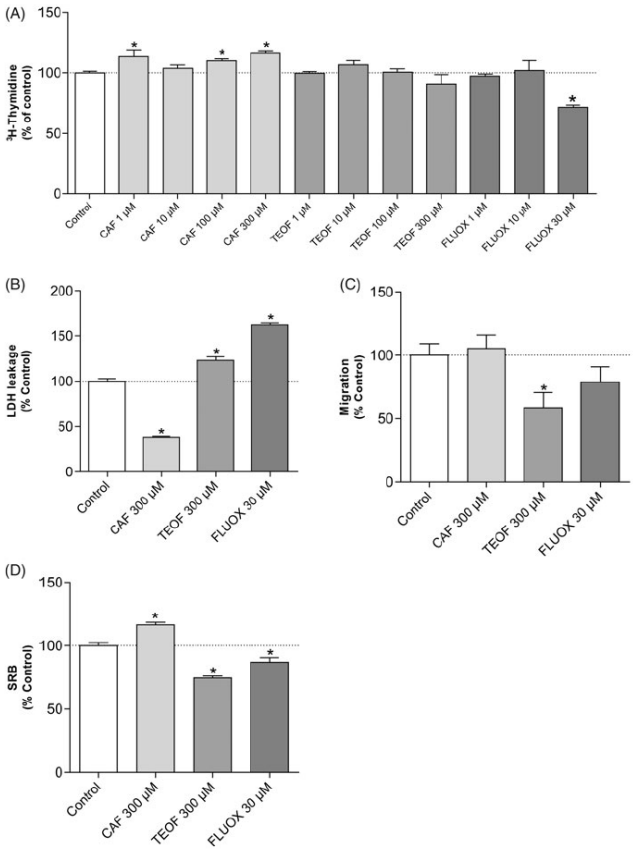

Figure 2. Characterization of the effect of methylxanthines and fluoxetine upon cell proliferation, cell viability, cell culture growth, and cell migration of HTR-8/SVneo cells. (A) Cells were exposed for 24 h to increasing concentrations of caffeine (CAF 1–300 μM; $n = 4$–8), theophylline (TEO 1–300 μM; $n = 4$–18), and fluoxetine (FLUOX 1–30 μM; $n = 4$) or the respective solvents (control, indicated by the dashed line; $n = 11$–35). (B–D) Cells were exposed for 24 h to caffeine (CAF 300 μM; $n = 12$–16), theophylline (TEO 300 μM; $n = 16$–18), fluoxetine (FLUOX 30 μM; $n = 8$–11), or the respective solvents (control, indicated by the dashed line; $n = 6$–23) and the effect upon cell viability (B), cell culture growth, (C) and cell migration (D) was evaluated. Shown are arithmetic means ± SEM. *$p < 0.05$ significantly different from control (Student's *t*-test).

selective serotonin reuptake inhibitors (SSRI) class. During pregnancy and in the immediate postnatal period, 10% of women present a major depressive disorder (Belik 2008), and it is estimated that 2–3% of pregnant women receive SSRI antidepressive treatment (Belik 2008). Our results, showing a detrimental effect of FLUOX on placentation-related parameters, are in accordance with our recent finding that FLUOX causes a significant impairment of nutrient (glucose) uptake by this cell line (Correia-Branco *et al.* 2015). It is worth of note that the concentration of FLUOX found to have a detrimental effect (30 μM) is higher than the usually observed therapeutic plasma levels (0.4–1.5 μM) (Moffat *et al.* 2004, Johnson *et al.* 2007), and so no direct extrapolation to the human *in vivo* situation should be made.

In summary, our results show a detrimental effect of the drugs of abuse AMPH, MDMA, of the methylxanthine TEO, and of the therapeutic drugs FLUOX in placentation-related processes of EVTs. Interestingly enough, COCA and CAF seems to favor HTR-8/SVneo cells placentation-related processes. Considering that the compounds tested have numerous cellular targets, it is vital in the future to comprehend how inhibition of placentation contributes to the detrimental effects of the herein studied xenobiotics.

Disclosure statement

The authors report no conflicts of interest.

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DISCUSSION AND CONCLUSIONS

CHAPTER I - Modulation of GLU transport and placentation-related processes in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

The results presented in **Chapter I** describe the functional characteristics of glucose uptake by HTR-8/SVneo cells, as well as its modulation by short-term exposure to xenobiotics. This chapter also includes the studied the modulation of glucose uptake by long-term exposure to XN and the impact in the process of placentation as assessed by the placentation-related processes viability, proliferation, culture growth and migration of HTR-8/SVneo cells. We additionally investigated the modulation of glucose uptake by long-term exposure to EtOH and METF.

To accomplish this, and as described in **Manuscript A**, we first characterized glucose uptake. For these experiments, ^{3}H-DG was used. This compound is a D-glucose analog efficiently transported by GLUT family members, but a very poor SGLT substrate [124], and it is not metabolized by phosphoglucose isomerase [125]. Our results indicated that ^{3}H-DG uptake by HTR-8/SVneo cells was as expected sodium-independent (see Introduction, section 2.1.), thus excluding the involvement of the SGLTs family. Moreover, ^{3}H-DG uptake by HTR-8/SVneo cells was mainly insulin-insensitive, saturable and inhibited by the GLUT inhibitors CYT B, PHT and PHZ. These characteristics led us to conclude that a GLUT transporter is the main mediator of glucose uptake by HTR-8/SVneo cells, that most probably corresponded to GLUT1 since this transporter is highly expressed in this cell line [126] and it is known to be critical for glucose uptake in placenta throughout pregnancy [21]. It is worth to note that although PHZ is classically described as a specific inhibitor of SGLTs [127], it has been described by our

group to inhibit also GLUTs [128, 129]. Because we here show that ^{3}H-DG uptake by HTR-8/SVneo cells was sodium-independent and excluded the SGLTs family, we can conclude that PHZ effect upon ^{3}H-DG uptake is related to GLUTs inhibition.

Next, given the importance of glucose in pregnancy (see Introduction, section 2.1.) and the scarce knowledge on glucose uptake modulation in first-trimester EVTs, we investigated modulation of ^{3}H-DG uptake by short-term (30 min) exposure to xenobiotics. As described previously (see Introduction, section 3.), the xenobiotics tested in the context of this study are frequently consumed during pregnancy [63, 64], and thus the study of their effects upon ^{3}H-DG uptake by the placenta is of major importance. These were: a) the therapeutic drugs FLUOX and DEXA; b) the drugs of abuse AMPH, MDMA, COCA, NICO and EtOH; and c) the dietary bioactive compounds EGCG, XN, QUE, RESV, CAF and TEO.

We observed that, among the compounds tested, FLUOX and DEXA reduced ^{3}H-DG uptake in a concentration-independent manner. A short-term exposure to the drugs of abuse AMPH, MDMA, COCA, NICO and EtOH did not affect ^{3}H-DG uptake by HTR-8/SVneo cells. Regarding the dietary bioactive compounds, the polyphenols QUE, EGCG, XN and RESV, and the methylxanthines CAF and TEO induced a concentration-dependent inhibition of ^{3}H-DG uptake. Of these, XN was found to be the most potent inhibitor of ^{3}H-DG uptake (IC_{50} of 3.55 μM). XN showed up to be a non-competitive inhibitor, since it decreased V_{max} without affecting the transporter affinity for ^{3}H-DG (K_m). These first results led us to conclude that ^{3}H-DG uptake by HTR-8/SVneo cells is susceptible to modulation by distinct therapeutic drugs and bioactive dietary compounds present in commonly consumed alcoholic and non-alcoholic beverages (e.g. beer, green tea and coffee). We further studied the effect of long-term exposure to XN upon ^{3}H-DG uptake, since this polpyphenol was the most potent inhibitor of the transport process. In a long-term

exposure to XN inhibited ^{3}H-DG uptake and, similarly to its short-term effect, it showed up to be a non-competitive inhibitor (decreasing V_{max} while not affecting the K_m of ^{3}H-DG uptake). This effect was associated neither with a decrease in GLUT1 mRNA expression levels nor with a decrease in GLUT1 protein levels, suggesting that the reduction of ^{3}H-DG transport induced by XN is related to changes in the intrinsic activity of the GLUT1 transporter. Furthermore, the inhibitory effect of long-term exposure to XN upon ^{3}H-DG uptake was found to be reversed by the tyrosine kinases (TKs) inhibitor genistein (10 mM), the c-Jun N-terminal kinases (JNK) inhibitor SP 600125 (5 mM) and mTOR inhibitor rapamycin (RAPA, 100 nM) but not by the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor LY 294002 (1 μM), thus indicating the involvement of TKs, JNK and mTOR intracellular pathways, but not PI3K, in the effect of XN. The fact both JNK and mTOR intracellular signaling pathways are both involved in the XN effect upon ^{3}H-DG uptake is not surprising as both have already been described to regulate each-other in synergy as a response to cellular stresses[130]. It is important to note that, as described in Introduction (section 4.), mTOR signaling pathway is considered a central nutrient sensor, is downregulated by glucose restriction [114] and is also downregulated in FGR placentas [103, 106]. Moreover, it is worth to note that to the present moment, very little is known regarding the effect of XN upon the here studied. Indeed, we found only a study by Deeb at. al., that shows that in prostate cancer cells, XN is associate with the inhibition of the intracellular signaling pathways Akt, NF-κB and mTOR, thus pointing to the same inhibitory effect we observed upon mTOR [131]. As such, our studies contribute to the knowledge of the effect of the polyphenol XN upon such important intracellular signaling pathways as mTOR.

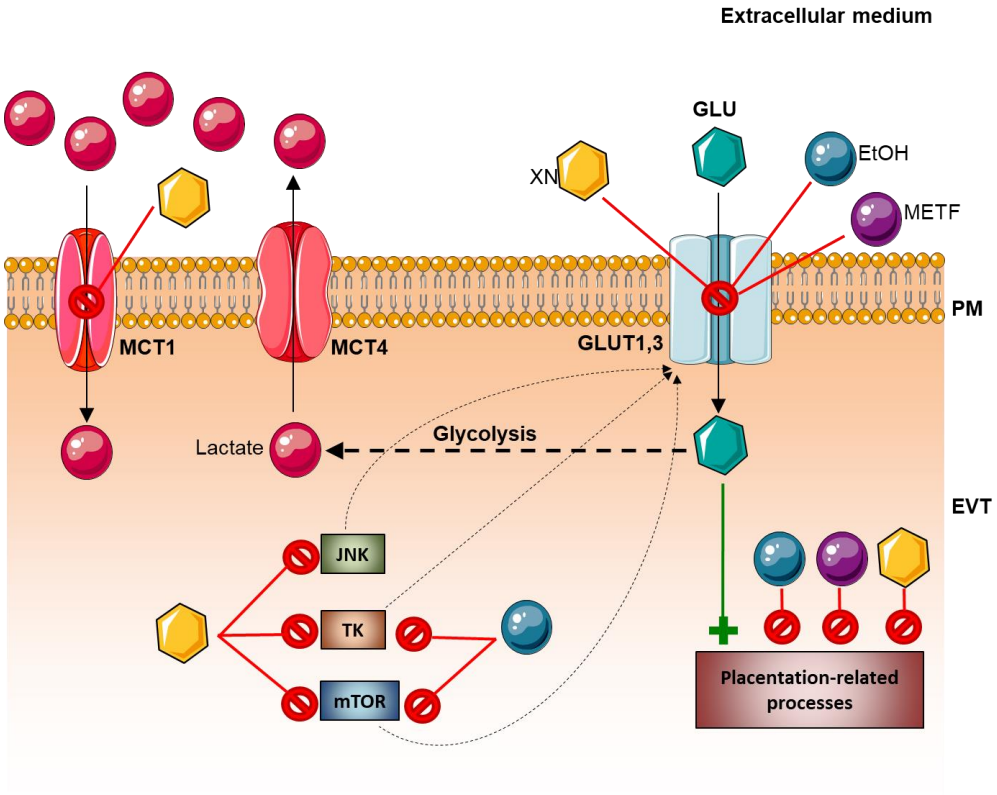

Fig. 13. Effect of xanthohumol, ethanol and metformin upon glucose uptake and placentation-related properties by HTR-8/SVneo cells.

Inhibitory effect of XN, EtOH and METF upon ^{3}H-DG uptake mediated by GLUT1/3. Lactate resulting from the process of glycolysis is transported to the extracellular medium by MCT4 and XN inhibits the re-uptake of lactate mediated by MCT1. XN inhibits TK, JNK and mTOR. EtOH inhibits TK and mTOR intracellular signaling pathways, which seems to be involved in the ^{3}H-DG uptake mediated by GLUT1/3. XN, EtOH and METF impair placentation-related properties whilst GLU seems to ameliorate these properties. EtOH: ethanol; EVT: extravillous trophoblasts; GLUT: glucose transporter; MCT: monocarboxylate transporter; METF: metformin; mTOR: mammalian target of rapamycin; PCFT: proton-coupled folate transporter; PM: plasma membrane; TK: tyrosine kinases; XN: xanthohumol.

Next, we observed that both short- and long-term exposure to XN induced an unexpected increase in extracellular lactate levels. These results are interesting as XN inhibited ^{3}H-DG uptake, thus, it would be expected a

reduction in a glycolysis product such as lactate unlike the results observed. As such, we hypothesized that XN could be impairing the reuptake of extracellular lactate by inhibiting the lactate transporter monocarboxylate transporter 1 (MCT1). In order to verify this, we studied the effect of XN on the uptake of the known MCT1 substrate butyrate. We confirmed that uptake of butyrate (^{14}C-BT) was significantly reduced after exposure to XN. Thus, we conclude that exposure to XN inhibits MCT1-mediated uptake of lactate, which may also starve cells of lactate (Fig. 13.).

Regarding the effect of XN upon placentation-related processes, we verified that a long-term exposure to XN impairs placentation-related processes such as cell viability, culture growth, proliferation and migration (Fig. 13.). Importantly, we showed that low extracellular glucose mimics the effect of XN upon cell viability and culture growth – *albeit* not its effect upon cell migration - and that high extracellular glucose reversed these nefarious effects of XN upon cell viability and culture growth. As such, we can conclude that the anti-proliferative and cytotoxic effects of XN are dependent upon glucose deprivation of HTR-8/SVneo cells. These results point to an interesting application of XN to induce EVTs insufficiency and thus to mimick FGR, as EVT insufficiency, characterized by shallow cellular viability, proliferation, migration, invasion and angiogenesis, is believed to be an underlying mechanism of FGR [132].

In the next part of **Chapter I**, as described in **Manuscript B**, we characterized the long-term effect of the drug the abuse EtOH and the therapeutic drug METF upon glucose uptake by HTR-8/SVneo cells. Alcohol use during pregnancy is associated with increased maternal and (or) fetal morbidity and mortality, higher risk of low birth weight, preterm delivery and teratogenesis [133, 134]. More alarming, in a prospective study from the years

2000 to 2012 including 5,036 pregnant women, 55% reported alcohol use in the first trimester [135]. We observed that long-term exposure (24 h) to EtOH (1-100 μM) concentration-dependently inhibited ^{3}H-DG uptake by HTR-8/SVneo cells. In line with this observation, previous studies from our group demonstrated that a 48h-exposure to EtOH reduced ^{3}H-DG uptake by the choriocarcinoma cell line BeWo [136]. Furthermore, the inhibitory effect of long-term exposure to EtOH (100 μM) upon ^{3}H-DG uptake was reversed by the mTOR inhibitor RAPA (100 nM) and by the JNK inhibitor SP600125 (5 μM), but not by the PI3K inhibitor LY294002 (1 μM) (Fig. 13.). This indicates the involvement of mTOR and JNK, but not the PI3K, intracellular signaling pathways in this EtOH effect. METF is widely used as first-line treatment in type 2 diabetes and PCOS [69] and is becoming increasingly accepted as an alternative to insulin during pregnancy, for the management of type 2 and gestational diabetes and PCOS [70-73]. Interestingly, we observed that long-term exposure (24 h) to METF (0.01-1 mM) concentration-dependently inhibited ^{3}H-DG uptake by HTR-8/SVneo cells (Fig. 13.). METF is described as an antihyperglycemic drug involved in the stimulation of GLU uptake by cells, thus METF inhibiting ^{3}H-DG uptake by HTR-8/SVneo cells is an unexpected result. However, it is of major importance to note that GLUT4 has been described to be activated by METF in order to increase the GLU uptake by human adipocytes [137], and as above described in **Manuscript A**, ^{3}H-DG uptake by HTR-8/SVneo cells is insulin-independent and is not mediated by GLUT4, thus providing an explanation to the fact that in this study METF inhibiting ^{3}H-DG uptake. These results point to a potential harmfulness of this therapeutic drug upon the placentation process and to insufficient nutrient supply to the placenta and the fetus. Furthermore, the effect of METF (1 mM) was not affected by mTOR, JNK and PI3K inhibitors, excluding the involvement of these intracellular signaling pathways in the effects of METF. These results are interesting as METF has previously been described to decreased

proliferation of human endometrial cancer by inhibiting PI3K/AKT/mTOR signaling [138]. Overall, these results are interesting, because as above described, mTOR signaling pathway is a central nutrient sensor, and mTOR seems to be involved in XN and EtOH long-term inhibitory effects upon glucose uptake by HTR-8/SVneo cells.

Interestingly enough, we observed **(Manuscript B)** that both EtOH, its metabolite ACA and METF presented a potent antiproliferative, cytotoxic and antimigratory effect in HTR-8/SVneo cells (Fig. 13.). Furthermore, as expected and as already described elsewhere for this cell line [139, 140], EtOH markedly induced cellular apoptosis of HTR-8/SVneo cells. METF also exhibited proapoptotic effect in HTR-8/SVneo cells. Altogether, these results suggest that both EtOH and METF present a harmfulness during pregnancy. However, it is not possible to confirm whether their inhibitory effect upon placentation-related processes here studied are dependent on inhibition of uptake of GLU and FA (as described in **Chapter I** and **Chapter III**).

CHAPTER II – Modulation of LC-PUFAs transport and placentation-related processes in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

Knowing the above described inhibitory effect of XN upon ^{3}H-DG uptake and upon placentation-related processes (cell viability, culture growth, proliferation and migration), we decided to investigate the effect of this compound on the placental uptake of other nutrients, as well as its implications upon the above mentioned placentation-related processes. So, the results presented in **Chapter II** describe the functional characteristics of the uptake of the essential nutrients ARA and DHA by HTR-8/SVneo cells, as

well as its modulation by short- and long-term exposure to XN. Next, we studied the modulation of ARA uptake by long-term exposure to XN and its impact in the process of placentation as assessed by the placentation-related processes viability, proliferation, culture growth, migration and apoptosis of HTR-8/SVneo cells.

In this part of the work, as observed in **Manuscript C,** we studied the effect of short-term exposure to XN upon ARA and DHA uptake by HTR-8/SV neo cells. Short-term exposure to XN inhibited the uptake of ^{14}C-ARA and ^{14}C-DHA in a concentration-dependent manner. In a more detailed characterization of the uptake of ^{14}C-ARA, we showed that this process was strongly depended on the activity of ACSL, as it was markedly inhibited by triacsin C (~75%), a potent inhibitor of ACSL activity [39]. These results are in good agreement with reported data obtained in primary cultured human term TBs and in cellular model of term human STB, the Bewo cells [37, 39, 40]. Although simple diffusion is considered quantitatively less important than the above referred protein-mediated transport of fatty acids [37, 141, 142], protonation of ^{14}C-ARA and thus simple diffusion of this fatty acid transport across the membrane lipid bilayer also appears to contribute to ^{14}C-ARA uptake by HTR-8/SVneo cells, because we observed an increase in ^{14}C-ARA transport with decreasing pH.

We next verified that long-term exposure to XN also inhibited ^{14}C-ARA uptake in a concentration-dependent manner. XN also inhibited ^{14}C-DHA uptake, but not in a concentration-dependent manner. Regarding the effect of a long-term exposure of XN upon ^{14}C-ARA uptake, XN showed up to be an uncompetitive inhibitor, as it reduced both V_{max} and K_m of ^{14}C-ARA uptake. Additionally, we verified that the intracellular signaling pathways TK, JNK and mTOR are involved in the inhibitory effect of XN upon ^{14}C-ARA uptake, similarly to what we described in **Chapter I** regarding glucose uptake [143]. Long-term exposure to XN also reduced the expression of ACSL1, an effect that

was prevented by high extracellular ARA (Fig. 14.), but not by the PPAR-γ agonist rosiglitazone (RG).

Next, in **Manuscript C**, we investigated modulation of ARA uptake by long-term exposure to XN and correlation of this effect with placentation-related processes such as cell viability, proliferation, culture growth, migration and apoptosis index. According to data already observed in **Manuscript A**, XN impaired placentation-related processes, by showing a cytotoxic, antiproliferative, antimigratory and proapoptotic effect (Fig. 14.). Importantly, all these effects were prevented by high extracellular ARA, but not by the PPAR-γ agonist rosiglitazone (RG). Interestingly, a long-term exposure of HTR-8/SVneo cells to XN leads to a similar phenotype as observed in insufficient EVTs from pregnancy-related pathologies such as FGR. So, XN exposure could provide a means to develop an experimental cell model of first-trimester insufficient EVTs. It is worth of note that, besides the culture of cell lines, it would be appropriate to study placentation-related processes such as EVTs proliferation, invasion and migration by using primary cell models, as isolated by the Kliman method [144]. This, however, is hampered by fact that: a) the amount of primary trophoblasts is limited; b) cultures may be contaminated with other placental cell types such as fibroblasts; and c) in Portugal, the access to normal first-trimester placentas is strongly limited. In Portugal, the termination of pregnancy is permitted according to the law number 16/2007, but when performed by the National Health System (Sistema Nacional de Saúde, SNS), the main procedure is mainly realized at home with legal medication, which invalidates either the viability of the explants and also the sterilized status required. As such, the experimental cell model of first-trimester insufficient EVTs that we here propose is, for the time being, the best approach to study EVTs insufficiency.

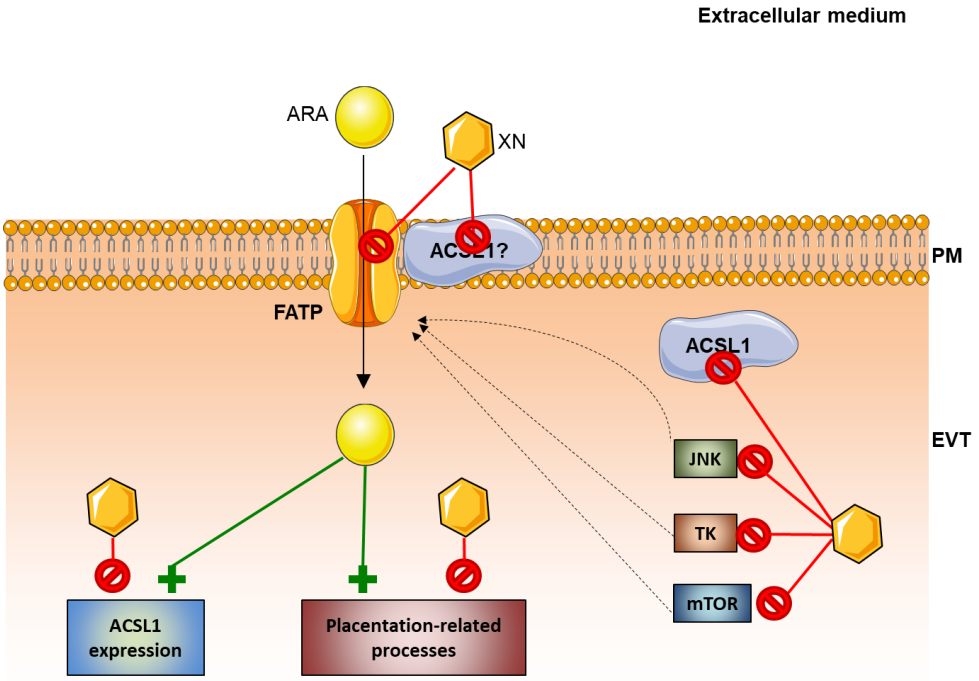

Fig. 14. Effect of xanthohumol upon arachidonic acid uptake and upon placentation-related properties on HTR-8/SVneo cells.

Inhibitory effect of XN upon ^{14}C-ARA uptake mediated by ACSL1. XN inhibits TK, JNK and mTOR intracellular signaling pathways, which seems to be involved in the ^{14}C-ARA uptake mediated by ACSL1. XN impairs placentation-related properties and ACSL1 expression whilst ARA seems to ameliorate these properties. ACSL1: long-chain acyl-CoA synthetases; ARA: arachidonic acid; EVT: extravillous trophoblasts; FATP: ATP-dependent fatty acid transport proteins; mTOR: mammalian target of rapamycin; PM: plasma membrane; TK: tyrosine kinases; XN: xanthohumol.

Altogether, the results regarding the effect of long-term exposure to XN upon nutrients uptake and modulation of placentation-related processes, obtained in **Chapter I** and **II** (with the results from **Manuscript A** and **Manuscript C),** led us to conclude that we have developed an experimental cell model of first-trimester insufficient EVTs. In fact, long-term exposure of

HTR-8/SVneo cells to XN lead to a similar phenotype as observed in insufficient EVTs from pregnancy related pathologies such as FGR. XN was able to impair the pro-placentation properties cell viability, proliferation, culture growth and migration and increased apoptosis in a GLU- or ARA-dependent manner, with involvement of TK, JNK and mTOR intracellular signaling pathways. We were also able to revert these detrimental effects by increasing the extracellular levels of these essential nutrients. This also points to the critical importance of both GLU and ARA during pregnancy, particularly during the first-trimester of pregnancy. Moreover, a central signaling pathway involved in the XN effects is mTOR, which also reinforces its key role as a nutrient sensor.

CHAPTER III – Modulation of FA transport in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

The results presented in **Chapter III** describe the functional characteristics of the uptake of folic acid by HTR-8/SVneo cells, as well as its modulation by short-term exposure to XN and its modulation by long-term exposure to XN, EtOH and METF.

To accomplish this, and as described in **Manuscript C**, we first characterized folic acid uptake by using ^{3}H-FA. Uptake of ^{3}H-FA was found to be markedly acidic pH-stimulated, suggesting that it involves the high-affinity folate: H^{+} symporter PCFT [145]. Accordingly, Keating et al. reported that FA uptake by human term CTBs obtained from normal pregnancies (NTB cells) is pH-dependent, operating optimally at acidic pH (5.0–5.5), and shows different transport characteristics at acidic (5.5) and physiological (7.5) pH [53]. This

point to a major putative role of PCFT as a placental FA transporter. We here hypothesize that, as the experiments were performed at acidic (5.5) pH, FA uptake might occur directly from the cell membrane, without further need of internalization of PCFT into an endosome (Fig. 15). Next, we verified that short-term exposure to XN inhibited ^{3}H-FA uptake in a concentration-dependent manner (Fig. 15.). However, long-term exposure to XN (24 h) did not consistently affect ^{3}H-FA uptake.

In the second part of Chapter III, and as described in Manuscript B, we characterized the long-term effect of the drug the abuse EtOH and the therapeutic drug METF upon folic acid uptake by HTR-8/SVneo cells. We observed that long-term exposure (24 h) to both EtOH (1-100 μM) and METF (0.01-1 mM) concentration-dependently inhibited ^{3}H-FA uptake. The inhibitory effect of EtOH upon ^{3}H-FA uptake is not surprising, as consumption of EtOH has been described to lead to FA deficiency and to both being associated with alcoholic liver disease (ALD)[146]. Also, the inhibitory effect of METF upon ^{3}H-FA uptake is not unexpected, as several studies show that patients treated with METF are at risk of FA and vitamin B12 deficiency [147]. Furthermore, the inhibitory effect of long-term exposure to EtOH (100 μM) upon ^{3}H-FA uptake was completely abolished by RAPA and reduced by SP600125 and by LY294002, indicating the involvement of mTOR, JNK and PI3K intracellular signaling pathways in this effect. Although we did not test for the putative involvement of Akt, we can hypothesize that this intracellular signaling pathway might also be involved, as it is classically described to belong to the PI3K/Akt/mTOR pathway. On the contrary, the effect of METF upon ^{3}H-FA uptake was not affected by these inhibitors, excluding the involvement of these intracellular signaling pathways in this effect. These results are in good agreement with the results observed for the long-term effects of EtOH and METF upon ^{3}H-DG uptake, described above.

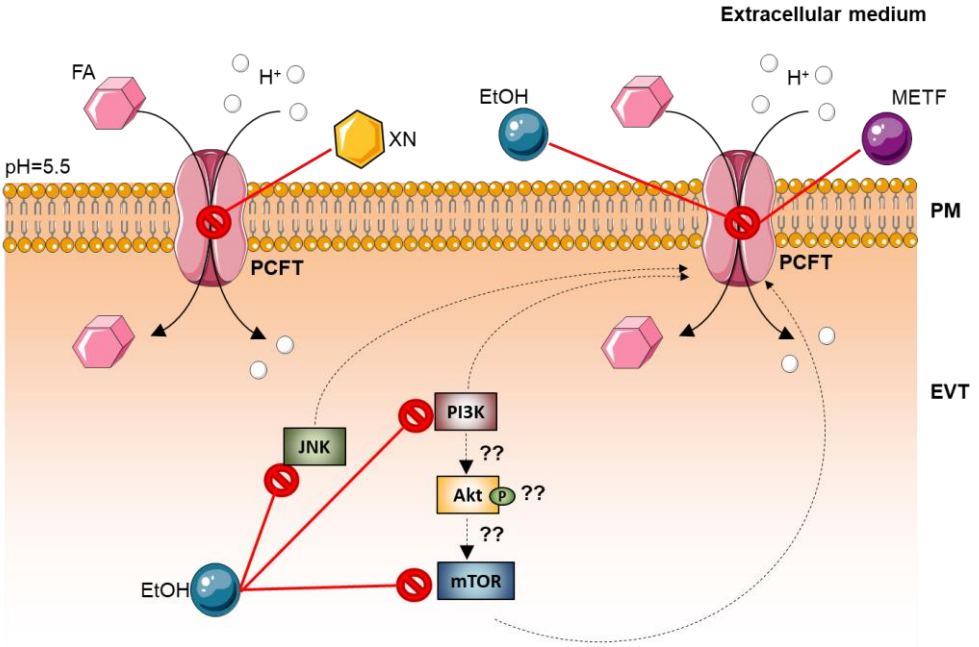

Fig. 15. Effect of exposure to xanthohumol, ethanol and metformin upon folic acid uptake by HTR-8/SVneo cells.

Inhibitory effect of XN, EtOH and METF upon ^{3}H-FA uptake mediated by PCFT. EtOH inhibits mTOR, JNK and PI3K intracellular signaling pathways, which seems to be involved in the ^{3}H-FA uptake mediated by PCFT. Akt is classically described as an intracellular signaling pathway downstream PI3K and upstream mTOR, thus we hypothesize that Akt might be also involved in the EtOH effect upon ^{3}H-FA uptake mediated by PCFT. Akt: protein kinase B; EtOH: ethanol; EVT: extravillous trophoblasts; FA: folic acid; H^{+}: proton; METF: metformin; mTOR: mammalian target of rapamycin; PCFT: proton-coupled folate transporter; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; PM: plasma membrane.

CHAPTER IV – Modulation of L-MET transport in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

Due to the above-mentioned inhibitory effect of XN upon GLU and ARA uptake, we also decided to investigate modulation of the uptake of the neutral amino acid L-Met by short- and long-term exposure to XN, and these results are presented in **Chapter IV.** To accomplish this, and as described in **Manuscript C**, ^{14}C-L-MET was used. Uptake of ^{14}C-L-MET by HTR-8/SVneo cells was shown to be time-dependent. A short-term exposure (26 min) of the cells to XN (1-500 μM) reduced ^{14}C-L-MET in a concentration-dependent manner. In contrast, a longer exposure (24h) of the cells to XN (0.03-5 μM) did not alter ^{14}C-L-MET uptake. Considering this, we did not proceed to further characterize the effect of XN upon the uptake of L-Met by HTR-8/SVneo cells.

CHAPTER V – Modulation of placentation-related processes in a human first-trimester EVTs cell line (HTR-8/SVneo cells)

In the last part of this work (**Chapter V**), we studied modulation of placentation-related processes such as cell viability, proliferation, culture growth, migration and apoptosis index by xenobiotics other than the above described (ie., XN, EtOH and METF).

We describe in **Manuscript D**, the effect of xenobiotics exposure upon placentation-related processes of first-trimester EVTs, and further unravel the putative involvement of the intracellular signaling pathways mTOR, cJNK and PI3K upon these xenobiotics effects. The compounds tested included the drugs

of abuse AMPH, MDMA, NICO, COCA, EtOH and its metabolite ACA, the methylxanthines CAF and TEO, and the therapeutic drugs FLUOX and METF.

The results obtained in this study with AMPH, MDMA, COCA and NICO reinforce the idea of the harmfulness of all these drugs of abuse, with the exception of NICO, to placental function and fetal health. Indeed, AMPH and MDMA presented an antiproliferative and cytotoxic effect; MDMA was also shown to have an antimigratory effect in HTR-8/SVneo cells. In contrast, COCA presented a proproliferative and cytoprotective effect. However, COCA also increased the cell apoptosis index. Usually, increased cell apoptosis is associated with reduced cell viability. Hence, we hypothesize that this increase in cell viability, proliferation and culture growth could be in response to the increased cell apoptosis index, thus, an increased cell turnover, in an attempt to maintain the cell population. It is worth of note that in our study, cell viability was determined by LDH assay, by measuring the release of the enzyme lactate dehydrogenase. Interestingly, a key signature for necrotic cells is the permeabilization of plasma membrane [148], hence the cell viability quantified in our study by LDH assay corresponds to the amount of cell necrosis. As well reviewed by Sten Orrenius et al. 2010 [149], apoptosis and necrosis are viewed as separate modes of cell death with distinct morphological characteristics, are controlled by complex signaling networks, comprising ‘‘switches’’ responsible for cross talk between them. Depending on the type of the cellular injury and on the severity (i.e. concentration) of the toxic agent under study, cell death in the form of either apoptosis or necrosis can be triggered. Most often, exposure to low doses results in apoptosis, whereas higher levels of the same toxic agent might cause necrosis [149, 150]. Indeed, several studies points to the fact that at lower doses of a toxic agent activation of apoptosis was observed, without observed alterations upon necrosis. In primary cultures of mouse proximal tubular cells, low concentrations of cisplatin (8 μM) led to apoptosis whilst only with higher

cisplatin concentrations (800 μM) cell necrosis was observed [151]. In LLC-PK1 renal proximal tubular cell line, low dose (4.2 nM) cyclosporine A induced apoptosis (quantified by TUNEL assay) whereas high dose (21 mM or greater) of cyclosporine A induced necrosis (quantified by LDH assay)[152]. Also, in the UVB-exposed HaCaT cells (human keratinocytes), extract of laver (*Porphyra yezoensis*) significantly increased the net viability and also the apoptotic cell [153]. As such, we can speculate that in our study, the COCA concentration was high enough to induce cell apoptosis but not as high to trigger cell necrosis, thus the increased cell viability (or reduced cell necrosis) observed. Another interesting hypothesis to the observation of increased cell apoptosis is the fact that classically, alterations in Ca^{2+} homeostasis can trigger or modulate an apoptotic response [149]. Interestingly enough, COCA is classically described to alter Ca^{2+} influx and storage in several brain cell lines. For instance, in rat brain microvascular endothelial cells (RBMVEC), COCA dramatically inhibits store-operated Ca^{2+} entry, a Ca^{2+} influx mechanism promoted by depletion of intracellular Ca^{2+} stores [154]. Therefore, we can theorize that in our study, COCA may affect Ca^{2+} homeostasis, thus enhancing an apoptotic response.

Overall, these results reinforce the idea of the harmfulness of drugs of abuse to placental function and fetal health, as these drugs are known to freely cross the placenta barrier, being associated with increased maternal and (or) fetal morbidity and mortality, higher risks of low birth weight, preterm delivery and teratogenesis [43, 45].

Interestingly enough, TEO was shown to present a cytotoxic and antimigratory effect, while CAF presented a stimulatory effect upon the placentation-promoting parameters tested. Regarding the therapeutic drugs, long-term FLUOX presented an antiproliferative and cytotoxic effect in HTR-8/SVneo cells, although it was devoid of effect on cell migration. This is

consistent with several studies that correlate the use of antidepressant during pregnancy with FGR and premature delivery [62, 155-157].

In summary, our results show a detrimental effect of the drugs of abuse AMPH, MDMA, and COCA, of the methylxanthine TEO and of the therapeutic drugs FLUOX in placentation-related processes of EVTs. Interestingly enough, CAF seems to favor HTR-8/SVneo cells placentation capacity. Nevertheless we understand that there is still some controversy concerning the risks associated with the maternal use of CAF during pregnancy (e.g. spontaneous abortion and fetal growth restriction), the FDA recommends that pregnant women should avoid the ingestion of CAF.

The final conclusions of this study are listed below.

1) GLU transport to HTR-8/SVneo cells involves GLUT, most probably GLUT1, and is affected by short-term exposure to different xenobiotics, including XN; long-term exposure to XN impairs placentation-related processes in a GLU-dependent manner.

2) GLU transport to HTR-8/SVneo cells is inhibited by long-term exposure to both EtOH and METF.

3) ARA transport to HTR-8/SVneo cells involves ACSL1 and is affected by XN, which impairs placentation-related processes in an ARA-dependent manner.

4) FA uptake by HTR-8/SVneo cells involves PCFT, and is affected by long-term exposure to EtOH and METF.

5) Long-term exposure to EtOH and METF impairs placentation-related processes.

6) Long-term exposure to XN impairs placentation-related processes and thus induces an insufficient phenotype in HTR-8/SVneo cells, which may contribute to the development of an experimental insufficient cellular model of EVTs.

Future perspectives

We believe that this work contributed for a better knowledge on the impact of xenobiotics upon the placental transport of nutrients and placental development during first trimester of pregnancy. However, it also showed that new aspects should be investigated in more detail, namely:

1) the modulation of placental transport of GLU upon HTR-8/SVneo cells by xenobiotics, including XN, and the putative involvement of GLUT3, as we did not observe statistically significant alterations upon GLUT1 gene expression and protein levels after XN exposure.

2) the involvement of intracellular signaling pathways on the XN effect upon placentation-related properties.

3) whether the observed effects of EtOH and METF upon placentation-related properties are dependent on GLU or FA.

4) the impact of XN upon placentation-related properties, placental and fetal development *in vivo*.

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"Home is now behind you, the world is ahead!"

~ J.R.R. Tolkien ~